THE EFFECT OF THE ESTROGENIC PHASE OF THE BOVINE ESTROUS CYCLE ON BLOOD LIPASE ACTIVITY

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CHAPTER I

INTRODUCTION

Although the gonadal steroids are preferentially concerned with the metabolism of the secondary sex organs, they exert a profound influence upon the metabolism of the entire mammalian organism. Estrogens have been shown to exert anabolic effects upon nitrogen and mineral metabolism in both experimental animals and in man. In recent years, there has been increased interest in the role of gonadal hormones in lipid metabolism with the demonstration in the human of the hypocholesterolemic actions of estrogens and the possible application of this to the treatment of atherosclerosis. Other studies have shown an estrogenic influence on the levels of other classes of circulating lipids in the blood of several species of animals. Various effects of estrogens on lipid metabolism have been reported indicating a wide variety of responses obtained both between and within species. Unfortunately, much of the data reported in the literature concerning the relationship between sex hormones and lipid metabolism appears to be inconsistent, making the establishment of definite conclusions difficult.

In recent years, general interest in the effects of gonadal hormones on the mammalian system has increased due to the more widespread use by humans of antifertility agents consisting of mixtures of progesterone-type hormones with estrogens. It has become obvious that a thorough knowledge of the many indirect effects as well as some possibly

unknown direct effects of continuous hormone administration is essential before its use as a method of contraception can be recommended. Since current knowledge of such effects in man and other species is somewhat limited, more investigation is needed to further establish the various changes brought about by estrogenic action, both during the regular sex cycle and during hormone administration.

This study was conducted to determine the effect of the estrous cycle on blood plasma lipase activity in the bovine. The changes in lipase activity were measured during normal estrus, estrus synchronization, and during estrogen administration to ovariectomized animals. The effect of estrogen administration on the level of circulating lipids was also determined.

CHAPTER II

REVIEW OF LITERATURE

Physical Characteristics and Nature of Control of Circulating Lipids

The plasma lipids consist principally of phospholipids, free and esterified cholesterol, triglycerides, fat-soluble vitamins, and a small quantity of free fatty acids. Most plasma lipids are conjugated with plasma proteins, the α and β-globulins, forming α and β-lipoproteins. According to Masoro (1968) the anhydrous composition of the lipoproteins of the highest density, the α-lipoproteins, is as follows: 45-55 per cent protein, $\sim$30 per cent phospholipid, $\sim$ 3 per cent cholesterol, $\sim$ 15 per cent cholesterol ester, and 5 to 8 per cent triglyceride. The low density lipoproteins or β-lipoproteins contain 20-25 percent protein, $\sim$ 22 per cent phospholipid, $\sim$ 8 per cent cholesterol, $\sim$ 35 per cent cholesterol ester, and $\sim$ 10 per cent triglyceride. Another broad class of lipoproteins has been called very low density lipoproteins or pre-β-lipoproteins. The chemical composition is highly variable and has been reported as follows: 2-13 per cent protein, 10-25 per cent phospholipid, 3-8 per cent cholesterol, 6-16 per cent cholesterol ester, and 50-80 per cent triglyceride. The other important class of plasma lipoproteins is the exogenous particles or chylomicrons. They are composed almost entirely of triglycerides with small amounts of protein, cholesterol and phospholipid. Plasma also contain free fatty acids,

most of which are bound to plasma albumin.

The exact mechanisms by which hormones act to maintain the concentration of these lipids in the plasma are not fully determined. Boyd and Oliver (1958) suggest that, since there is no evidence for the oxidative catabolism of lipids in the extracellular fluid, the concentration of the circulating lipoproteins would appear to be dependent upon the following factors: (1) changes in the rates of transference between the extracellular and intracellular lipoproteins, such as can result from alteration in the rate of lipid synthesis, storage, mobilization, degradation or excretion; (2) changes in the distribution of lipoprotein between the plasma and the interstitial fluid at the capillary endothelial level; or (3) changes in the plasma volume. Hormones could affect the concentration of plasma lipoproteins by influencing any or all of these factors.

Sex Differences in Levels of Circulating Lipids

The earliest evidence indicating an influence of sex hormones on lipid metabolism was the clinical observation that the occurence of coronary atherosclerosis was much less frequent in post menopausal women than in men of a comparable age. It was also observed that there was little or no difference in the incidence of the disease in post menopausal women compared to men. The apparent correlation of ovarian function with the atherosclerotic condition led to the investigation of possible sex differences in plasma lipids at various ages. In recent reviews Hess (1964) and Marshall (1969) reported that Russ et al. (1951) found that women between the ages of 18-35 had lower total serum cholesterol concentrations than men of comparable age. They also reported

that these women had lower β-lipoprotein and higher α-lipoprotein cholesterol than the men. They found no significant difference in levels of these lipids in men and women of older ages. A study by Oliver and Boyd (1953) found that, during the menstrual cycle in healthy young women, the plasma cholesterol, the plasma cholesterol:phospholipid ratio and the β-lipoprotein cholesterol were lower at ovulation, when endogenous estrogen secretion was maximal, than at any other time during the cycle. No similar cyclical depression of these lipids was observed in normal young men. Elevation of plasma cholesterol and inversion of the β/α -lipoprotein cholesterol ratio has been observed by Oliver and Boyd (1958) in the first post-menopausal decade in normal women. In addition, Oliver and Boyd (1959) noted an elevation of serum cholesterol in women after bilateral ovariectomy. However, Hess (1964) reports that Ritterband et al. (1963) were unable to confirm this observation. Also, Havel et al. (1955) found no difference in serum cholesterol concentration between the sexes, although the serum of the women examined tended to have more lipid in the high-density fractions, while the men had more in the lower density lipoprotein fractions.

Although some of the above reports appear contradictory concerning sex differences in the concentration of various serum lipid classes in man, many of the observations indicate an estrogenic influence tending to lower the levels of some lipids, notably cholesterol and low density lipoproteins.

Studies concerned with sex differences in serum lipid levels of experimental animals have been generally less extensive and primarily restricted to observations on cholesterol and phospholipid levels.

Lorenz et al. (1938) found that mature female chickens had higher concentrations of neutral lipid, phospholipid and cholesterol in the serum than male birds. Wood et al. (1961) later confirmed this by demonstrating a higher concentration of serum cholesterol in mature female chickens than in males of a comparable age.

Fillios and Mann (1956) reported a similar relationship in rabbits. They found that, in contrast to humans, male rabbits had significantly lower average serum cholesterol levels than females both before and after the feeding of a high-cholesterol diet. In addition, castration appeared to lower the cholesteremic response in the females, while it raised the cholesteremic response of the males during the period of high-cholesterol feeding.

Sex differences in serum lipid levels in the rat have been more thoroughly investigated. Fillios (1957) found that adult female rats had significantly higher levels of serum cholesterol than adult males. Fillios et al. (1958) later reported that this sex difference is not observed until about the age of puberty. They also reported that the highest female levels were found during the follicular phase of the estrous cycle, with a significant peak appearing during the proestrus to estrus phase. A sex difference in other plasma lipids of rats fed an essential fatty acid diet was found by Lyman et al. (1966). They reported that female rats maintained higher concentrations of arachidonic acid and stearic acid in plasma phospholipids than did male rats. However they found no sex difference in concentrations of plasma cholesterol esters, phospholipids and triglycerides, or in the fatty acid composition of plasma triglycerides.

Effects of Exogenous Estrogen Administration

Indirect evidence of possible estrogenic effects on lipid metabolism offered by sex differences in plasma lipid levels resulted in investigations concerning the possible therapeutic value of estrogen administration for atherosclerosis. Eilert (1953) obtained a slight but significant decrease in total serum cholesterol and an increase of phospholipid level in a group of postmenopausal women upon subcutaneous administration of estradiol. In a group of male survivors of myocardial infraction, Russ et al. (1955) found that the percent total cholesterol in the α-lipoprotein fraction of the blood was lower than normal, while that in the β-lipoprotein fraction was higher than normal. Administration of ethinyl estradiol corrected this trend and decreased the total cholesterol levels in all hypercholesterolemic patients. This decrease was seen in both free and esterified cholesterol fractions.

Estrogen administration has produced somewhat variable results in laboratory animals. Roseman et al. (1952) found no significant alteration in serum cholesterol levels after estrogen injections in normal rats. However, Fillios (1957) obtained elevated serum cholesterol concentrations in castrated male and female rats upon administration of estradiol dipropionate. Lyman and Krueger (1961) reported a definite lipotropic action by estrogen injection in both normal and castrate male rats. They noted a decrease in liver glyceride lipid and an increase in plasma cholesterol and phospholipid over nontreated controls. Lyman et al. (1966) also found that estradiol caused a significant rise in plasma phospholipids and triglycerides during a period of feeding an essential fatty acid-deficient diet to castrated rats. In this study, intact and castrated female rats injected with estradiol appeared to

have consistantly more free cholesterol than either intact or castrated males treated with testosterone. Fox et al. (1961) measured total cholesterol, phospholipid and neutral fat in the serum of male rats fed diets containing varying amounts of fat and cholesterol. They found that injection of estradiol in the rats fed a low-fat diet resulted in an increase in phospholipid and neutral fat levels and a decrease in total serum cholesterol levels. The estrogen injections also produced a depression of serum cholesterol concentrations in rats fed intermediate and high-fat diets. Laron and Kowadlo-Silbergeld (1965) also reported an increase in plasma free fatty acids following estrogen injection in rats which were starved for three days.

The influence of sex hormones and diet on lipid metabolism in the rat was also investigated by Aftergood and Alfin-Stater (1965). They reported that plasma cholesterol levels of females fed a control-diet were not significantly affected by either gonadectomy or gonadectomy followed by estrogen administration. However, in the rats on a fat-free diet, estrogen administration caused an increase in the plasma cholesterol level, as well as an increase in the content of arachidonic acid in plasma lipids.

Fewster et al. (1967) determined the effect of estradiol benzoate on the concentration of lipids in the plasma, liver and heart of male rats fed a normal diet. They found that estrogen injection resulted in a significant decrease in plasma total lipid concentration from 254 to 161 mg/100 ml. The mean plasma phospholipid and both free and esterified cholesterol concentrations were also reduced. No significant changes were found in the plasma tri- or diglyceride or free fatty acid fractions, but a significant decrease was reported in the monoglyceride

concentration.

Sufficient variation in experimental conditions could possibly account for the apparent variety of results and many of the contradictions reported in the literature concerning the relationship between sex hormones and lipid metabolism. In addition to the age and nutritional status of the experimental animals, a number of other environmental factors can affect the homeostatic state of plasma lipid levels in the mammalian organism. Many of these factors exert their influence through a multiplicity of hormones which can alter lipid metabolism at one or more sites, i.e. absorption, synthesis storage, mobilization, degradation and excretion.

Estrogenic Influence on Lipid Biosynthesis

Compared to the extensive investigations concerning the effect of sex hormones on serum lipid concentrations, relatively few studies have been conducted to determine the relationship between the estrogens and hepatic lipid biosynthesis. These studies have also yielded widely varying and conflicting results. Endogenous synthesis of cholesterol from C^{14}-labelled acetate, as indicated by C^{14} activity of serum cholesterol, is reported by Fillios et al. (1958) to be greater in female rats than in males of comparable age. They also report that the acetate-to-cholesterol conversion is reduced in ovariectomized rats, and that this conversion is enhanced by estradiol, but not by progesterone. In support of this, Aftergood and Alfin-Slater (1965) report that female rats showed a significant reduction in the rate of conversion of acetate to liver cholesterol following ovariectomy and that estradiol treatment restored it to the original rate. Contradictory evidence is offered by

Rosenman et al. (1952), who measured the biliary concentration of cholesterol as an index of the rate of hepatic synthesis and found that estradiol depressed the rate of hepatic cholesterol synthesis. In a more recent study, Fewster et al. (1967) found that intact male rats treated with 1.7 mg of estradiol benzoate per day for five days had a diminished incorporation of the label from C^{14}-acetate in vivo into free and esterfied cholesterols, glycerides, free fatty acids and phospholipids of the liver. On the other hand, Perry and Bowen (1958) reported that estradiol pretreatment of female rats given at a rate of 1 mg per day for ten days had no appreciable effect on the rate of conversion of labelled acetate to hepatic cholesterol by liver slices in vitro while conversion of the label to hepatic and adipose tissue fatty acids was enhanced. Humber et al. (1962) demonstrated that estrone inhibits in vitro cholesterol biosynthesis from mevalonate-2-C^{14} by an enzyme system prepared from homogenates of rat liver. Direct comparisons of the results of the in vivo studies employing exogenous hormones are not valid because of the diversity of experimental conditions employed.

Studies concerning the effect of estrogens on phospholipid synthesis have yielded generally more consistent results. Ranney and Weiss (1958) investigated the effect of the administration of estrone, estradiol benzoate, and the synthetic steroid methoxymethyl estratriendiol upon the rates of incorporation of inorganic P^{32} into the phospholipids of liver and plasma in cockerels. They concluded that the resulting increase in phospholipid concentration was a reflection of increased turnover rate, since enhanced synthesis of phospholipid exceeded the accelerated rate of disappearance of phospholipid from the circulation. Phospholipid turnover rate was also increased in the liver, with leci-

thin synthesis proceeding more rapidly than that of the cephalins. Bowser et al. (1961) conducted a similar study with ovariectomized rats to determine the role played by estrogen in the prevention of nutritionally induced fatty liver. Estradiol increased the hepatic lecithin concentration in the animals which were fed a high fat, low choline diet and produced a significant lipotropic effect. However, the estrogen did not affect the hepatic lecithin concentration or the rate of inorganic P^{32} incorporation into lecithin in the rats which were on the control diet.

Increases in phospholipid synthesis were demonstrated by Aizawa and Mueller (1961) with in vivo and in vitro experiments involving the treatment of the rat uterus with estradiol. The administration of 10 mg estradiol to ovariectomized rats resulted in an increase in endogenous uterine synthesis of ethanolamine, choline and inositol, as indicated by increased tissue concentrations of these phosphatides by as much as 150 per cent. Furthermore, pretreatment of ovariectomized rats with a single injection of 10 mg estradiol enhanced incorporation of inorganic orthophosphate-P^{32} into the individual phospholipid fractions as much as 200 per cent faster than the uterine segments from control rats. Significant increases were also achieved in the rate of incorporation of acetate-1-C^{14} into fatty acids, cholesterol and nonsaponifiable fractions of uterine sections, but reflected a different time course than that observed for the phospholipid labeling. It was concluded that the action of the hormone involved some step which is common or fundamental to the synthesis pathways of all these lipid classes, possibly that of induction of synthesis of certain enzymes.

This hypothesis received further support by Gorski and Nicolette

(1963) who, in a similar study, found that the in vivo incorporation of orthophosphate-P^{32} occurred in the RNA of subcellular fractions of the rat uteri within the first hour after estradiol-17β injection. This preceeded the incorporation of the isotope into phospholipid. This was later confirmed by Gorski et al. (1965) who also reported that the estrogen-stimulated increase in glucose metabolism and increase in lipid and RNA synthesis were blocked by inhibitors of RNA and protein synthesis at a time when no effect on overall protein synthesis was noted, suggesting that the synthesis of specific enzymes may be involved.

The Influence of Hormones on Lipid Mobilization

Another important means by which the levels of circulating lipids could be altered is that of affecting change in the rate of release of lipids from the adipose tissue stores of the body. Adipose tissue consists primarily of triglycerides. When mobilization occurrs, the triglyceride molecules are hydrolyzed by a system of lipases into glycerol and free fatty acids which diffuse into the blood and are delivered by the cardiovascular system to the tissues which require fuel. The rate limiting step in the lipolysis is the reaction catalyzed by an enzyme which has been called "hormone-sensitive lipase." According to Masoro (1968) the hormone-sensitive lipase is active in the adipose tissue of fed animals, but is much more active in that of fasting animals. Therefore, the adipose tissue of fasting animals hydrolyzes more triglyceride to free fatty acids and glycerol than the tissue of the normal animal, which is in a state of dynamic equilibrium, with free fatty acids and glycerol being continuously deposited and released resulting in little

net loss or accumulation. In the fed state, adipose tissue is capable of metabolizing glucose to L - α glycerophosphate, which is utilized with free fatty acids to synthesize triglycerides. Therefore, most of the free fatty acids which are liberated by the hydrolysis of triglycerides are retained in the tissues and used again for synthesis of triglycerides. Consequently, there is little or no net release of free fatty acids. However, in the fasting state very little plasma glucose is available for metabolism by the adipose tissue, thus decreasing the availability of L - α -glycerophosphate for the synthesis of triglyceride, and leading to accummulation, then release of free fatty acids from the tissue. Therefore, conditions leading to an increased rate of lipid mobilization do so either by increasing the activity of the adipose tissue hormone-sensitive triglyceride lipase or by decreasing the rate of trigylceride biosynthesis in the adipose tissue, or by both mechanisms.

Hormone-sensitive lipase was first discovered by Gorden and Cherkes (1958) and White and Engel (1958), who independently demonstrated that when epinephrine was added to rat adipose tissue in vitro, it stimulated the liberation of free fatty acids. Rizack (1961) extracted the enzyme from the rat epididymal fat pad and, upon characterization, determined the epinephrine-sensitive lipase to be identical to the lipolytic enzyme which responds to fasting. Further investigation of the enzyme by Hollenberg et al. (1961), Buckle (1962), Vaughn and Steinberg (1963) and Lewis and Matthews (1968) showed that lipolysis of mammalian adipose tissue could also be stimulated by norepinephrine, glucagon, adrenocorticotropic hormone (ACTH), α and β melanophore stimulating hormone (MSH), luteotropic hormone (LTH), thyroid-stimulating hormone

(TSH), and growth hormone (GH) in addition to epinephrine.

Further insight into the fat mobilization process was gained by Vaughan et al. (1964) who demonstrated the presence of a monoglyceride lipase in rat epididymal fat pads. This enzyme could be distinguished from hormone-sensitive triglyceride lipase by differing chemical properties. Furthermore, its lipolytic activity was not affected by incubation with norepinephrine, whereas such incubation of the tissue increased the hormone-sensitive triglyceride lipase activity 2- to 3-fold. Strand et al. (1964) also demonstrated the presence of monoglyceride and diglyceride lipase activities in the rat adipose tissue. This confirmed other studies which showed that monoglyceride lipase activity and hormone-sensitive lipase activity could be dissociated, and further indicated that diglyceride lipase activity could be differentiated from hormone-sensitive lipase activity. However, they were unable to dissociate the activities of the two lower glyceride lipases, so whether they are the same or different enzymes is not yet known. These findings indicate that the first step in the lipolysis of triglyceride to free fatty acids and glycerol is the reaction catalyzed by the hormone-sensitive triglyceride lipase in which the triglyceride is converted to a free fatty acid and a diglyceride, which in turn is degraded to glycerol and free fatty acids by another lipase or lipases.

Although the specific mechanism for hormonal influence in increasing the rate of lipolysis of adipose tissue triglycerides has not been definitely established, it is generally believed to be through the activation of an inactive form of the hormone-sensitive lipase found in the adipose tissue cell. According to Butcher (1966), the catecholamines (and possibly the pituitary peptide hormones, glucagon, etc.) stimulate

the adenyl cyclase system and thus increase intracellular cyclic adenosine 3', 5'-monophosphate.

Since Rizack (1964) has found that lipase activity in a fraction of homogenized adipose tissue can be increased by the addition of cyclic adenosine 3', 5'-monophosphate plus ATP, it is thought that this increase in activity is brought about by the conversion of an inactive form of the enzyme to an active form, similar to the action of cyclic adenosine 3', 5'-monophosphate on phosphorylase systems of other tissues, i.e. liver and skeletal muscle.

Besides the well know pituitary hormones previously mentioned, several less well characterized pituitary peptides have been found which have the ability to promote lipid mobilization. Friesen et al. (1962) isolated two peptides from a crude extract from the anterior pituitary glands of hogs which stimulated the release of free fatty acids from rabbit adipose tissue in vivo and in vitro. These peptides had only a slight effect in promoting lipolytic activity in man. Rudman et al. (1963) compared the adipokinetic activity of one of these peptides, called fraction H, with several other hormones in the rabbit, guinea pig, hamster, rat, pig and dog. They found that ACTH, TSH, α-MSH, β-MSH, arginine vasopressin, fraction H, epinephrine and norepinephrine each had high adipokinetic activity in certain species and no detectable activity in others. Ryshka and Khokhlow (1965) extracted a polypeptide from pituitary glands of the ox, swine and sheep, which exhibits lipotropic activity, yet is reported to be distinguished from other pituitary hormones. Birk and Li (1964) obtained a peptide from sheep pituitaries which they call lipotropin. A bovine anterior pituitary extract called Fraction G, which had been extracted by Rudman et al. (1961) was repor-

ted by Holland (1969) to be inactive in the rat and man under normal conditions, but was found to promote free fatty acid release from adipose tissue after fasting. The data available concerning these lipid mobilizing hormones are too fragmentary at this time to permit significant comment in regard to their biochemical mechanisms of actions and physiological significance.

Another lipase, or lipase system, which has become the subject of much study in recent years, is called clearing factor lipase or lipoprotein lipase. Discovery of this lipase resulted from the report by Hahn (1943) that administration of heparin to dogs with turbid plasma due to alimentary lipemia resulted in a marked clearing of the turbid plasma. Korn (1955) isolated and characterized the enzyme, and found that it catalyzes the hydrolysis of triglycerides of plasma chylomicrons upon activation or release by heparin. It is generally beleived to function in the uptake of circulating lipids, the hydrolyzed free fatty acid esters, by tissues. Bezman et al. (1962) measured the incorporation of C^{14} labeled triglyceride fatty acids into slices of adipose tissue from rabbits and found that it was positively correlated with the lipoprotein lipase activity released from the tissue under the influence of heparin. Garfinkel et al. (1967) simultaneously measured lipoprotein lipase activity and the uptake of labeled lipoprotein triglyceride by the rat epididymal fat pad in vivo and in vitro. The lipoprotein lipase activity was stimulated by injecting fasted rats with actinomycin D or fed glucose. They reported a correlation coefficient between enzyme activity and C^{14} uptake of 0.81 ($P < 0.01$) from the actinomycin-treated rats and 0.75 ($P < 0.01$) from the glucose-fed rats. They concluded that lipoprotein lipase plays a major role in trigly-

ceride deposition in adipose tissue.

The exact location of the enzyme and mechanism of heparin stimulation have not yet been established. Ho et al. (1967) reported that, while epinephrine-sensitive lipase seemed to be localized in the intracellular compartment of fat cells, the heparin-released was associated with the stromal-vascular cells of the adipose tissue. However, Rodbell (1964) and Nestel et al. (1969) demonstrated lipoprotein lipase activity in extracts of isolated fat cells. In addition, Patten and Hollenberg (1969) reported that intact fat cells released lipoprotein lipase activity when incubated with heparin, whereas the intact stromal-vascular cells did not. They also found that the increase in lipoprotein lipase in the presence of heparin was not due to the preservation of enzyme activity by the heparin, but to increased binding of the enzyme to chylomicrons. In an attempt to determine the relationship between heparin and lipoprotein lipase, Naito and Felts (1970) investigated its inactivation by the liver. They proposed that the hepatic inactivation by destruction of heparin by a heparinase and (2) removal of the apoenzyme of the lipoprotein lipase. They suggest that the circulating enzyme consists of a complex of apoenzyme, heparin and a serum component which acts enzymatically on a triglyceride substrate, which has been activated with the same or a different serum fraction, possibly α-lipoprotein.

Another interesting recent development in the investigation of lipoprotein lipase is the report by Greten et al. (1969) that a separate monoglyceride hydrolase exists along with triglyceride lipase in post-heparin plasma of the human. They found that the activity of both the enzymes appeared and disappeared at approximately the same rate. How-

ever, when compared to the triglyceride lipase, the monoglyceride lipase activity was much greater, less heat sensitive, uneffected by triglyceride lipase inhibitors, and was not affected by radical changes in the fat or carbohydrate content of the diet.

The influence of gonadal hormones on lipoprotein lipase activity has not been determined. However, there has been some indirect evidence that a hormonal influence possibly exists. Robinson (1963) found that the lipoprotein lipase activity of the lactating mammary gland tissue of the guinea pig was much higher than any other tissue of the animal which had been studied. He reported that the lipase activity in the mammary gland remained constant during pregnancy but increased to some 100 times greater at parturition. McBride and Korn (1963) confirmed this, reporting that the concentration of lipoprotein lipase increased markedly just prior to parturition, reaching its maximal level within two hours post-partum. The activity remained until within 18 hours after the cessation of suckling. Later McBride and Korn (1964) correlated this activity with the uptake of free fatty acids in the mammary tissue. They found that both free fatty acids and chylomicron glycerides from the plasma were taken up by mammary gland tissue of lactating guinea pigs approximately 20 times more rapidly than by tissue from midpregnant animals. These studies show that that activity of lipoprotein lipase increases under conditions when the uptake of triglycerides by the tissues is increased, and in the mammary tissue, a time when the gland is in a state of high functional activity and hormonal influence.

Lipase and Lipid Studies in the Bovine

Although as estrogenic influence on lipid metabolism has been de-

monstrated in several mammalian species, very little research has been conducted to determine the effect of estrogen on plasma lipid levels on lipase activity in the bovine. Several workers have offered evidence of an effect of estrus on some constituents of bovine milk. Aurand et al. (1968, p. 1296) studied the hormonal influence on the development of oxidized flavor in milk as measured by the 2-thiobarbituric acid (TBA) method. Milk samples from four cows which had normal estrous cycles were tested, and an increase in TBA values was found on or near the day of onset of estrus in each cow. In addition, when estradiol was injected into a normal cow, a cystic anestrus cow and two ovariectomized cows, a consistent increase in TBA values was noted. Aurand et al. (1968, p. 1862) measured the fatty acid composition of milk from a cow which had been injected with 5 mg estradiol benzoate for five days. They reported an increase in the 18-carbon unsaturated fatty acids and the branched fatty acids and a reversal in the percent composition of the 16- and 18- carbon saturated fatty acids. They concluded that the estrogen had little or no effect on the fatty acids which were synthesized by the mammary gland, whereas the fatty acids known to arise from the blood glycerides were affected.

It has been thought for several years that lipase activity in milk might be related to rancidity problems in milk, since rancidity is believed to be caused by the presence of free fatty acids in the milk. Kelly (1945), searching for clues to the rancidity problem, found that lipase activity of milk was influenced by the estrous cycle. He measured the lipase activity in 420 milk samples from five cows and consistently found the greatest lipase activity a few days before the onset of heat, followed by a drop in activity just before heat and another

increase during or immediately following heat.

There is also some evidence that the lipase activity of blood is influenced by the estrous cycle. Roussel and Stallcup (1966) investigated the possibility that the lipase activity level could be used as an indicator of heat in cattle. Using nine cows, they reported a highly significant ($P < .01$) difference between lipase activity in the follicular phase and the luteal phase of the cycle, but concluded that the lipase activity was of little value in detection of heat. Haggerty (1966), in pursuit of a similar objective, determined the effect of the stage of estrous cycle on the level of blood plasma lipase activity of four cows. He found that the level of lipase activity was significantly ($P < .01$) higher at estrus than during proestrus or metestrus. However, levels were quite variable during diestrus, which negated the use of lipase level as an indicator of the heat stage of the cycle. Wells et al. (1969) measured the lipase activity in both the blood and milk in four normal, cycling cows. Their results agreed with Haggerty's (1966) in that peak lipase activity in blood occurred about 24 hours before observed estrus. These lipase activities were at a low level two or three days before estrus, increased to a peak value within 24 hours before estrus, then decreased to the previous low level one to three days after estrus. A similar change occurred in one cow during a cycle which included a silent heat period. They also reported that peak lipase activities were found in milk nine to 15 hours after they were observed in the blood.

These studies indicate that lipase activity follows very closely the urinary estrogen pattern observed at estrus by Mellin and Erb (1966). Therefore, they offer an indication that there is a possible estrogenic

influence on lipid metabolism in the bovine as has been reported in laboratory animals. If such an estrogenic influence exists, it could be mediated through a lipase or system of lipases, affecting the rate of mobilization of lipids or uptake of lipids by the tissues, resulting in an alteration of the levels of circulating lipids.

CHAPTER III

MATERIALS AND METHODS

Treatment and Sampling of Experimental Animals

Trial I.

Six normal, cycling, non-lactating Holstein cows were selected from the Oklahoma State University dairy herd for blood plasma lipase study. Blood samples (50 ml samples with 2.9% sodium citrate added as an anticoagulant) were taken by juglar puncture twice weekly for a period of five weeks. During this period the animals were confined in a single pen and observed daily for signs of estrus activity. This preliminary sampling period allowed the determination of normal levels of lipase activity for each animal.

Following this initial sampling period, a more intense sampling procedure was used. The animals were bled daily for a period of 15 days. During this period, at the first sign of estrus activity, each animal was bled at four-hour intervals for 24 hours. This phase of the trial was intended to give a daily lipase activity value for each animal before and after heat, as well as a more complete picture of changes in lipase activity during the heat period. Previous studies by Haggerty (1966) had indicated that more frequent sampling was necessary to reveal the rapidly changing levels from proestrus through metestrus.

Trial II.

Phase 1. Eight normal Ayrshire heifers of approximately 18 months of age were selected from the dairy herd for an extended study of lipase activity patterns. The animals were kept together in one pen and observed daily for signs of estrus activity. Blood samples were collected three times each week for a period of five weeks. In addition, samples were collected twice daily from each animal on the day when heat was detected. The animals were group fed a normal ration of hay and silage.

Following these initial observations, the eight heifers were placed in individual stalls and fed a daily ration of approximately 15 pounds of alfalfa hay and three pounds of a concentrate grain mixture. After a period of four weeks in which the animals were allowed to become accustomed to the stalls, the heifers were fed the estrus synchronizing compound, melengestrol acetate (MGA), at a rate of 1.0 mg per head per day for 15 days. The compound was thoroughly mixed with the grain concentrate when each animal was fed to insure complete consumption. Blood samples were collected daily at the beginning of the MGA treatment, then twice weekly for the balance of the 15 day period. Beginning the last day of MGA feeding, samples were collected three times daily (at 9 a.m., 3 p.m., and 9 p.m.). On the second and third day post-treatment, samples were collected four times daily at six-hour intervals in order to obtain a more accurate measure of variation in lipase activity around the time of expected estrus. On the day following estrus, sampling frequency was decreased to three times daily for four days. Twice weekly sampling was continued until 18 days after the majority of the exhibited estrus. At this time the sampling frequency was increased

again to three or four times daily at six-hour intervals, in order to measure the lipase activity variation around this expected heat period. After five days of frequent sampling, the trial was concluded. This study should give an accurate indication of variations in lipase activity before, during and after estrus for each animal.

Phase 2. Following the above treatment the eight heifers were ovariectomized and allowed three months time for recovery. They were again placed individual stalls and fed a similar hay and concentrate ration as used in the previous trial. Four of the animals were randomly assigned to a treated group and the other four animals served as a control group. Each animal in the treated group received injections of 5.0 mg estradiol benzoate dissolved in 1.0 ml ethyl oleate daily for five days. The control animals received similar injections of the carrier substance without the hormone. The injections were made at nine o'clock each morning and blood samples were collected at 9 a.m., 1 p.m. and 5 p.m. each day. The animals were not fed until after the last blood collection each day to prevent the effect of alimentary hyperlipemia on the blood lipase activity. In addition to measurement for blood lipase activity for each animal, total lipid levels and levels of several lipid fractions were measured in the blood of two animals from each group. With the ovarian influence removed, the effect of the specific hormone on plasma lipase activity and changes in the levels of various classes of circulating lipids could, therefore, be measured.

Laboratory Procedures

Determination of Plasma Lipase Activity

Blood was collected in tubes containing sodium citrate as an anticoagulant and immediately cooled to 0°to 5° C. The blood samples were then centrifuged at 5000 rpm for 15 minutes to obtain plasma. Lipase activity was determined according to a modification by True (1969) of procedures reported by Kern et al. (1961) and Rizack (1961). Duplicate 50 ml erlenmeyer flasks were prepared, each containing 5.0 ml of blood plasma, 5.0 ml of tris buffer at pH 8.5 and 2.0 ml of a tributyrin substrate solution. The tributyrin substrate consisted of 7.5 g tributyrin (>99% pure)[1] and 15.0 g bovine serum albumin diluted to 100 ml with water and emulsified in a hand-operated laboratory emulsifier. The samples were incubated in a shaking water bath for one hour at 37° C. The reaction was then stopped by adding 10% HCl to reduce the pH of the mixture to <2.0. The duplicate blank of each sample was treated in a similar manner except the acid was added to the mixture prior to the plasma and the blank was not incubated.

Free butyric acid which was released during the reaction was extracted twice with a 1:2:2 mixture of ethanol, ethyl ether and petroleum ether, then quantitated by titrating with 0.02 N alcoholic potassium hydroxide using thymolphthalein indicator. This extraction procedure is reported by Jack et al. (1963) to recover 90% of the butyric acid. The

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[1]Less than 1% free fatty acids by titration, balance assumed to be glycerides. Sample saponified with KOH and methyl (esters of released fatty acids identified as >99% butyric acid by gas-liquid chromatography.

data were expressed as lipase units per milliliter of plasma, with a lipase unit being defined as the amount of enzyme required to hydrolyze one micromole of butyric acid per minute of reaction time. Preliminary investigation had shown that excess substrate was present in these reaction mixtures, maintaining first-order kinetics during the entire reaction time.

Determination of Plasma Lipid Levels

Additional blood samples were collected in Phase 2 of Trial II from randomly selected animals, two from each group, for determination of blood lipids. Plasma was obtained from these samples in the manner previously described. Duplicate 15 ml plasma samples were obtained from each animal. Lipids were extracted twice with a 3:5:5 mixture of ethanol, ethyl ether and petroleum ether at room temperature. The extracts were dried in tared beakers and quantitated gravimetrically. The lipid classes were separated by chromatography on Florisil according to modifications of a procedure described by Carroll (1961). The Florisil used in this study was 60 to 100 mesh material activated at 1200° F. It was deactivated by adding 7 ml water per 100 g of Florisil and allowed to stand for at least 12 hours to equilibrate.

The separations made in this study were carried out with columns containing 12 g of Florisil. The columns were prepared by filling the chromatographic tubes with petroleum ether and slowly adding the dry Florisil. The excess ether was then allowed to flow out until the surface of the liquid reached the top of the packed column. The lipid samples were then added to the top of the columns in a small volume of petroleum ether and the head of liquid again was lowered to the level of the column.

Preliminary experimentation with known standards of triglycerides, cholesterol and free fatty acids, as well as with lipid extracts from bovine plasma, indicated that maximum separation of the classes could be obtained with eluting solvent volumes slightly altered from those reported by Carroll (1961). The following schedules of elution solutions were used in this study:

Fraction	Eluent	Eluting Solvent	Volume of Solvent (ml)
I	Hydrocarbons	petroleum ether	20
II	Cholesterol esters	4% ethyl ether in petroleum ether	75
III	Triglycerides	15% ethyl ether in petroleum ether	80
IV	Cholesterol	25% ethyl ether in petroleum ether	76
V	Diglycerides	50% ethyl ether in petroleum ether	60
VI	Monoglycerides	2% methanol in ethyl ether	75
VII	Free fatty acids	4% acetic acid in ethyl ether	75

The eluent from the columns was collected as whole fractions in tared beakers. The solvents were removed by evaporation and the lipid residues determined gravimetrically. Confirmation of triglycerides in Fraction III was accomplished by detection of glycerol according to a procedure reported by Clark (1964). Cholesterol in Fractions II and IV

were characterized by the Salkowski Test also reported in Clark (1964). In addition, each major fraction was compared with individual reference compounds in its position as it was eluted from the column.

CHAPTER IV

RESULTS AND DISCUSSION

Six holstein cows were used for the blood lipase study in Trial I. In the first phase of the trial, blood samples were collected twice weekly for one month while the cows were being observed to determine that they were cycling. A graphical representation of the lipase activity values for these animals during these collection periods are shown in Figure I. It can be seen that, although the lipase values of the group were generally within a range of .090 to .160 lipase units, the lipase activity in some individuals tended to fluctuate rather widely. Cows 906, 933 and 957 had lipase values that remained fairly constant over the sampling period. On the other hand, the lipase values of cows 930, 932 and 945 varied to more extreme ranges and appeared more erratic. The days in which each cow was observed in estrus are marked with an asterisk (*). From inspection of these patterns it would appear that estrus had very little if any effect on lipase activity. However, since these blood samples were collected at three- and four-day intervals, the detection of possible changes in lipase activity immediately before and after estrus was impossible. Therefore, definite conclusions concerning the effect of estrus on lipase activity could not be reached without more frequent sampling.

The second phase of this trial involved frequent sampling during the heat period to determine as clearly as possible the relationship be-

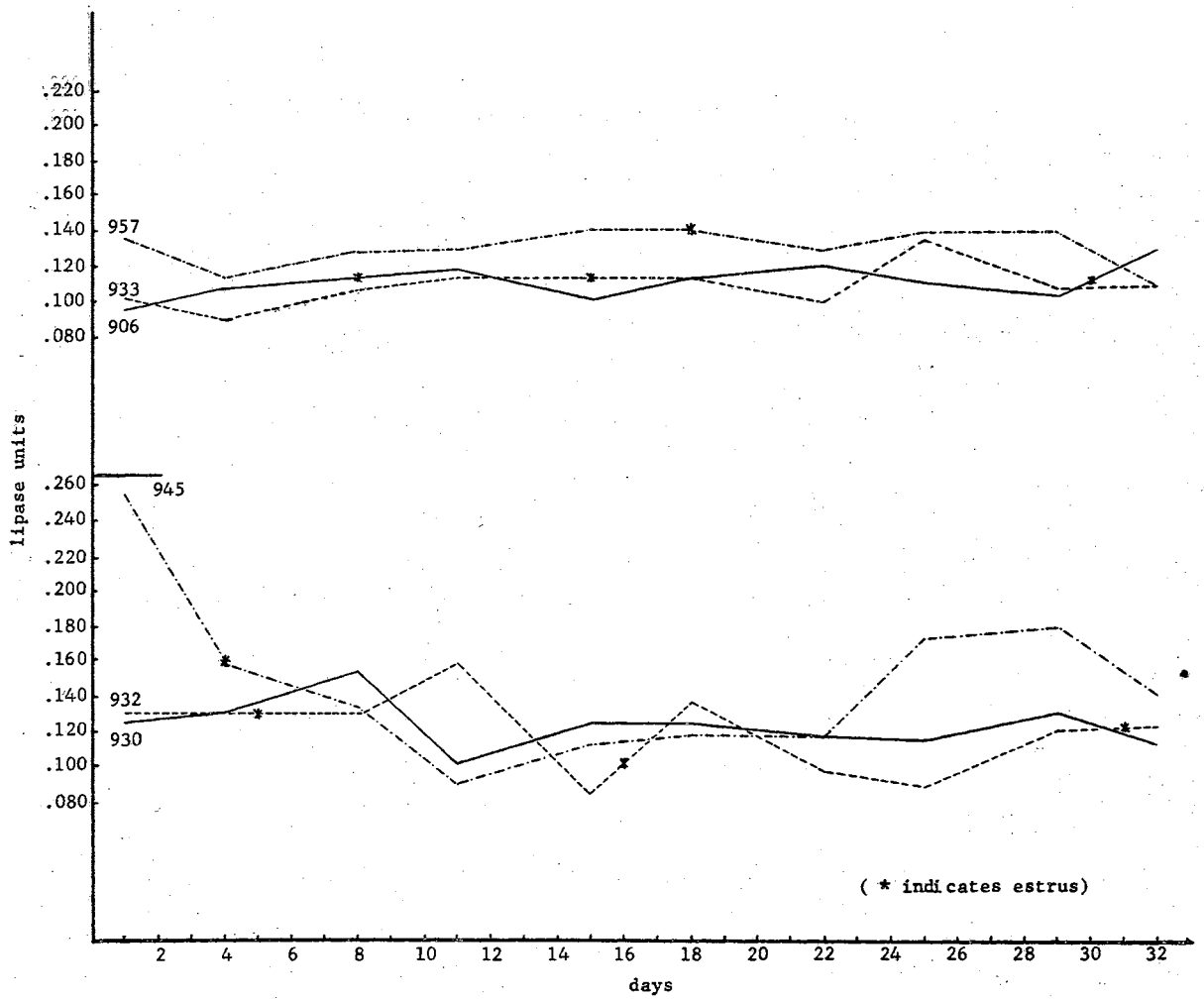

Figure 1. Lipase activity expressed as lipase units (u moles of butyric acid/min) per milliliter of plasma for six cycling cows during a 32-day period.

tween heat and the lipase pattern. Since one cow did not exhibit heat during this phase, she was excluded from this part of the experiment. Blood samples were collected daily from each of the five other cows. When each cow began to show the earliest signs of heat, blood samples were collected at four-hour intervals for 24 hours. When the resultant lipase activity values are plotted according to the day in which estrus began, as shown in Figure 2, the similarity of the patterns becomes apparent. Unfortunately, samples on the days preceeding estrus were not obtained from two of the cows. However, in cows 930, 932 and 957 there was a consistent peak in lipase activity on the day prior to heat. In all the cows there was a peak on the day of estrus. Beginning the day of estrus each animal had a sharp decrease in lipase activity followed consistently by another peak usually of lesser magnitude. The activity then appeared to decline and increase again, usually more gradually. These patterns were very similar to the milk lipase activity patterns around estrus reported by Kelly (1945) and the milk and blood lipase activity changes described by Wells et al. (1969).

The lipase activity values for the eight heifers in Phase 1 of Trial II are shown in Figure 3 and Figure 4. These graphs represent the changes in lipase activity in each animal during the five week preliminary study and during the 15-day MGA treatment. As previous studies have shown, there was much variation in lipase activity which was not associated with heat. However, in most cases in which heat was detected, there were indications of a similar pattern as was found in Trail I. Again, the lack of frequent sampling preceeding, during and after heat did not permit the definition of the characteristic peaks. The lipase activity patterns during the MGA feeding generally tended to be less

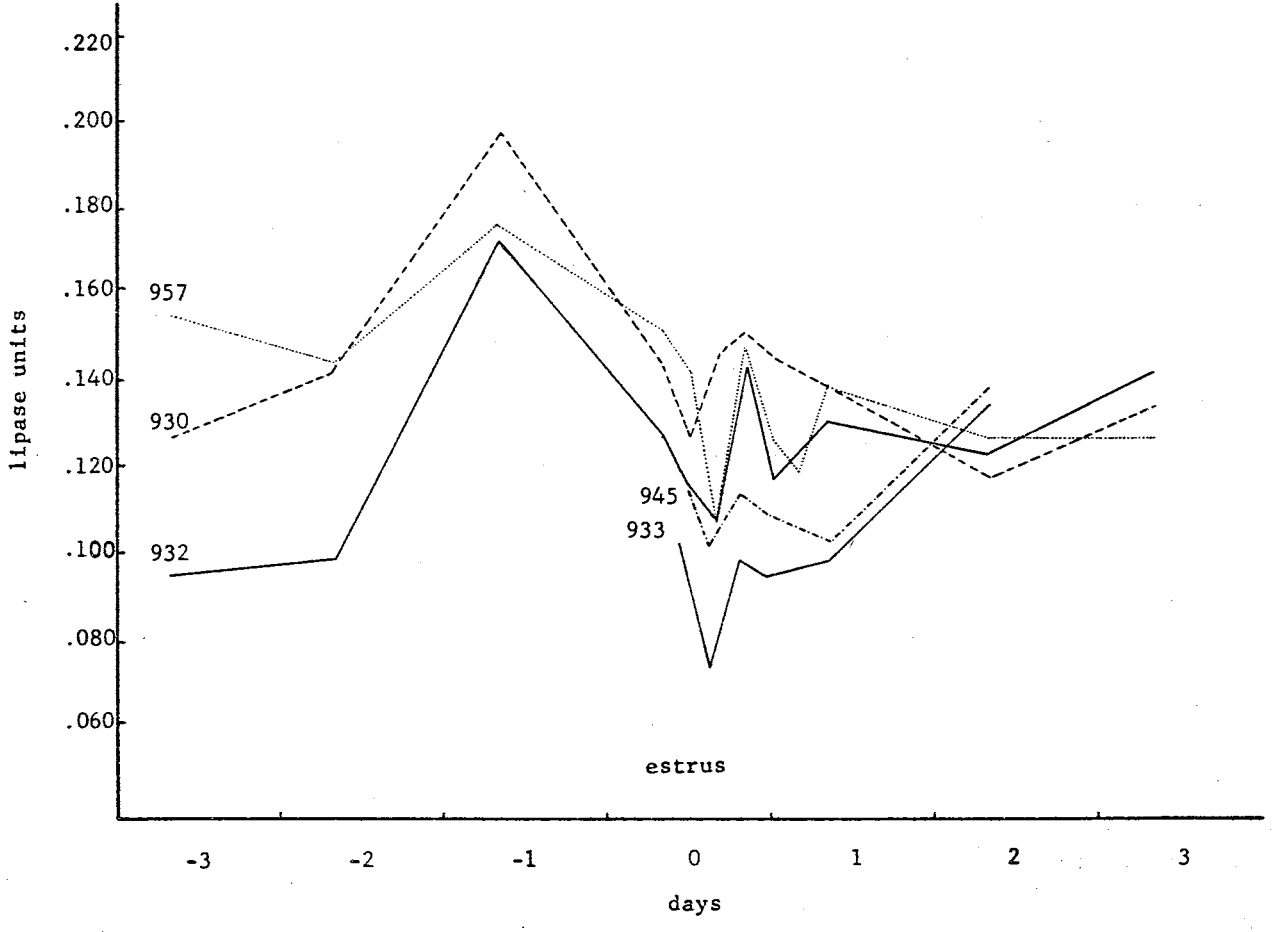

Figure 2. Lipase activity (lipase units per ml of plasma) for five cows during estrus.

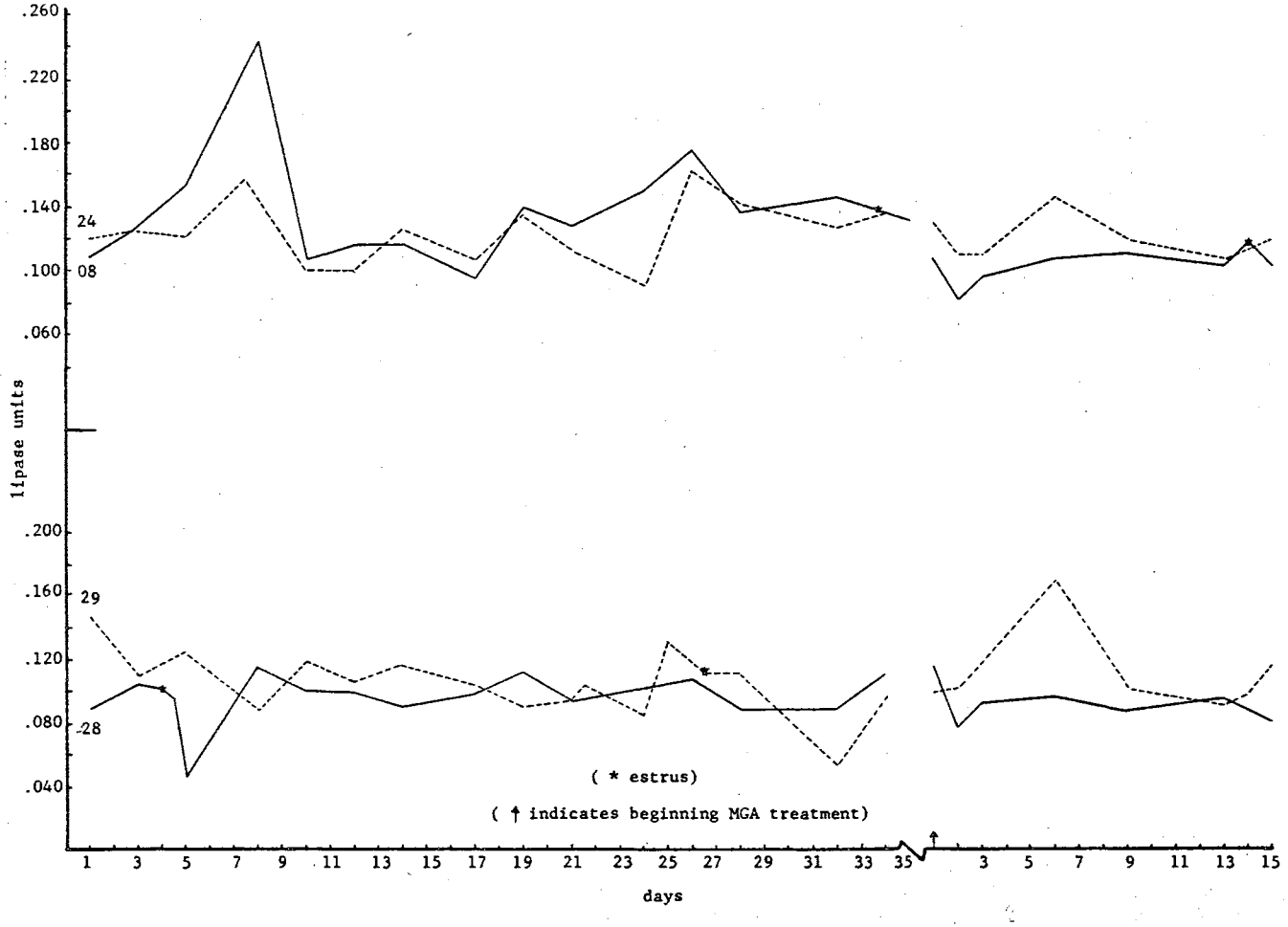

Figure 3. Lipase activity (lipase units per ml of plasma) for Heifers 08, 24, 28 and 29 during preliminary observation and during MGA treatment.

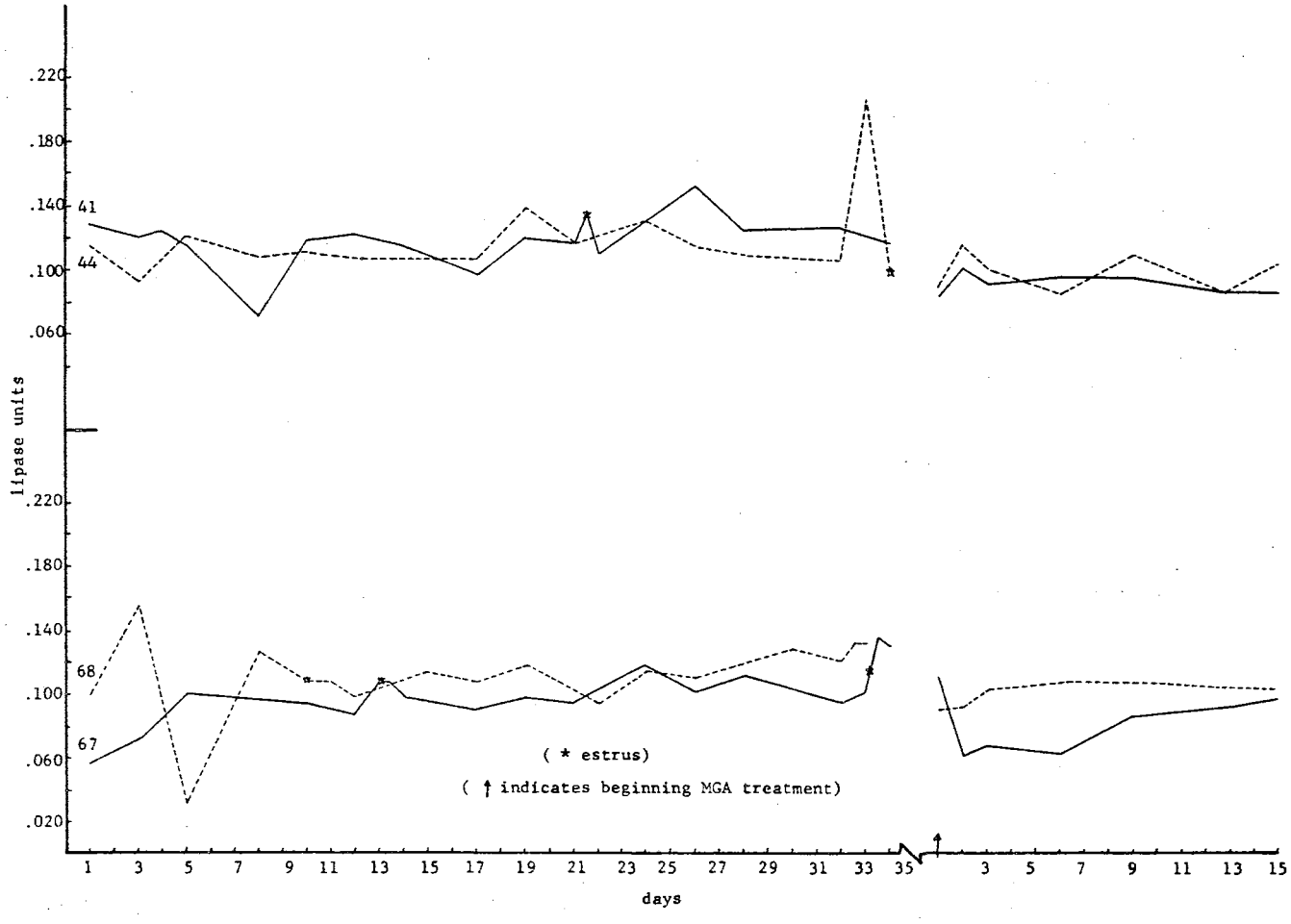

Figure 4. Lipase activity (lipase units per ml of plasma) for Heifers 41, 44, 67 and 68 during preliminary observation and during MGA treatment.

variable than during the previous period. This could be expected since the hormone treatment temporarily delayed the onset of estrus and ovulation and arrested the cyclical nature of ovarian function. This is consistent with the findings of Wells et al. (1969), who observed that blood lipase activities were less variable during pregnancy than prior to conception.

The patterns of lipase activity associated with the first estrus after removal of the MGA are shown in Figures 5, 6 and 7. Data were not available from heifer 08. This animal came in heat earlier than expected due to the accidental removal of MGA from her ration after 12 days of the treatment. Also, the data from heifer 41 was not included because estrus was not detected in this animal during the sampling period. These lipase activity patterns obtained during the first estrus after MGA removal, as well as those observed during the second heat, shown in Figure 8 and Figure 9, continued to have the same general characteristics as had been seen before in naturally cycling animals. In most cases, an obvious peak occurred either one or two days prior to the first visible signs of estrus. This was usually followed by a decline in lipase activity, then another increase during estrus. Statistical comparison of the lipase activity in the samples collected at the first visible sign of estrus and the samples collected six hours earlier, according to the method for paired observations described by Steel and Torrie (1960) and shown in Table I, revealed a highly significant difference ($P < .01$). This increase in lipase activity was usually followed by a decline, then a gradual increase for several days. Although the patterns were generally similar, they are obviously quite variable in magnitude and duration of peaks of activity. During the first heat

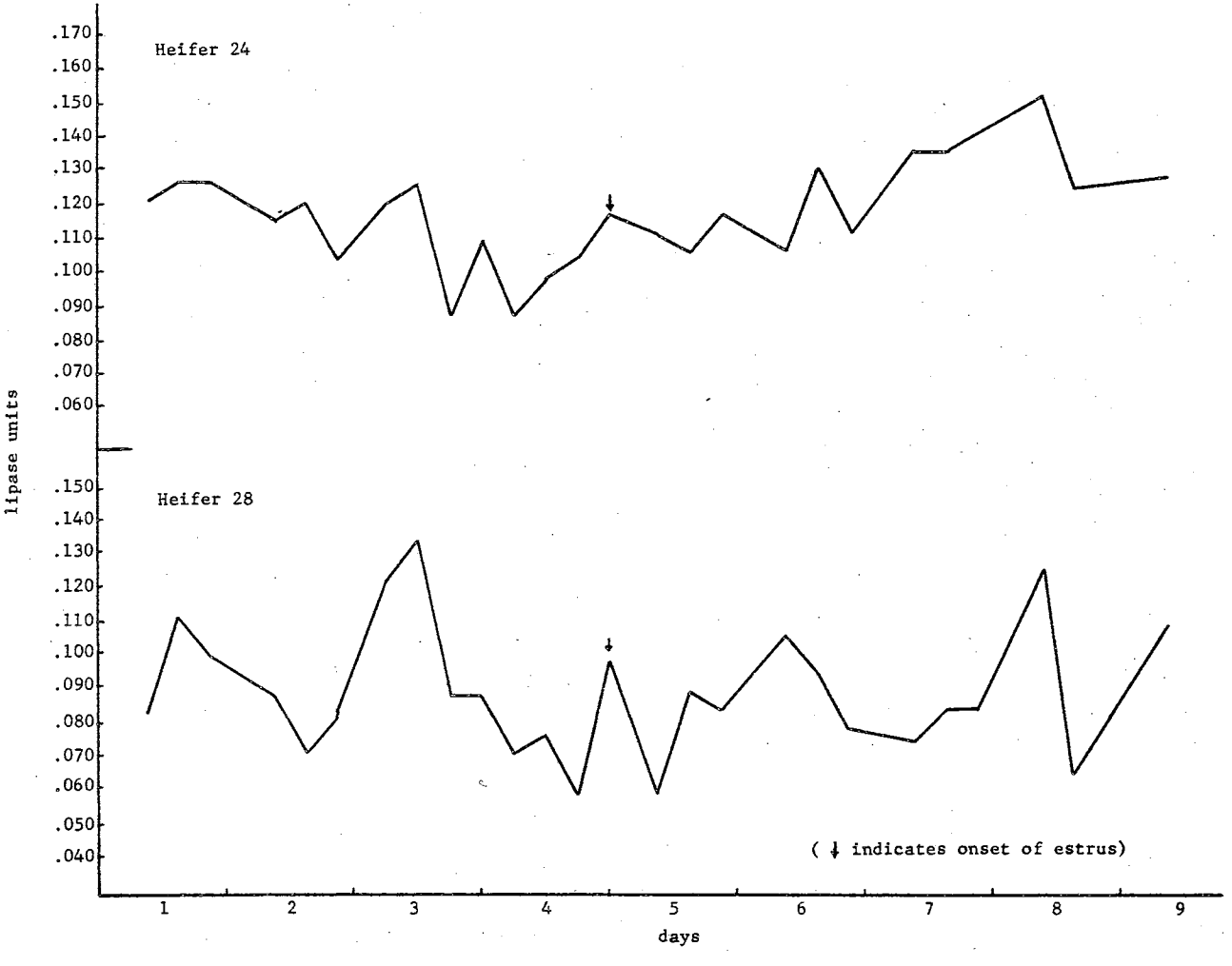

Figure 5. Lipase activity (lipase units per ml plasma) of Heifers 27 and 28 during the first estrus following MGA treatment.

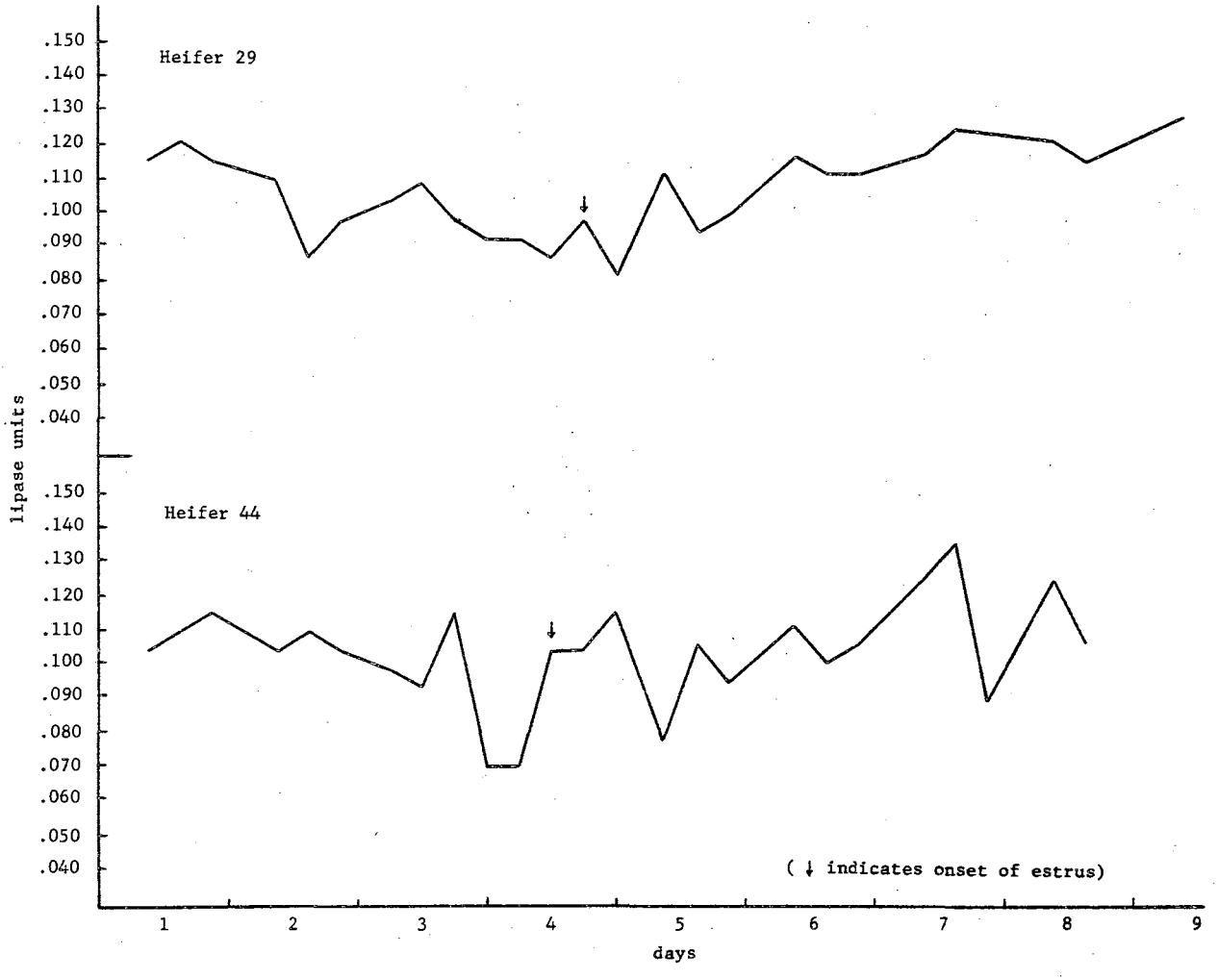

Figure 6. Lipase activity (lipase units per ml plasma) of Heifers 29 and 44 during the first estrus following MGA treatment.

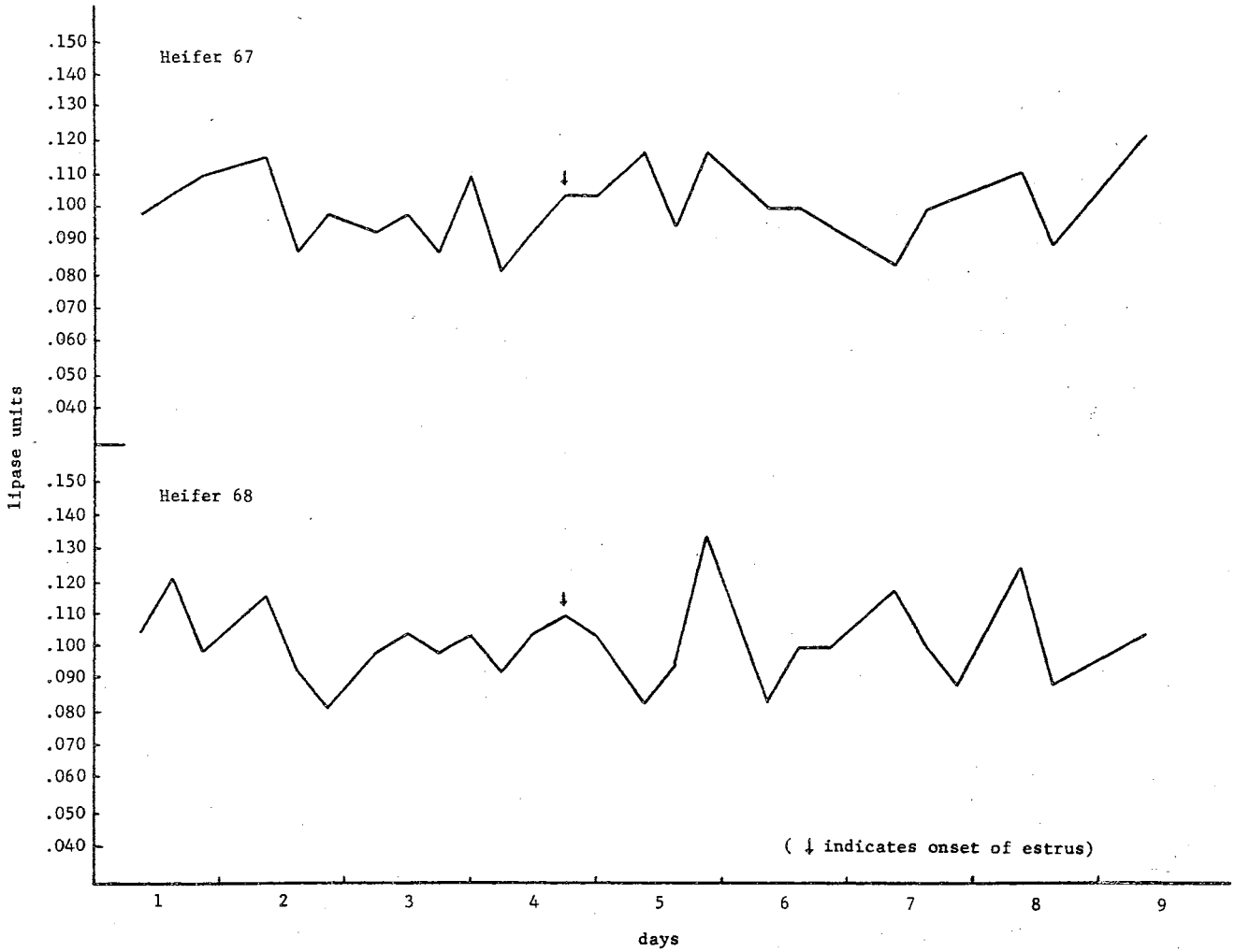

Figure 7. Lipase activity (lipase units per ml plasma) of Heifers 67 and 68 during the first estrus following MGA treatment.

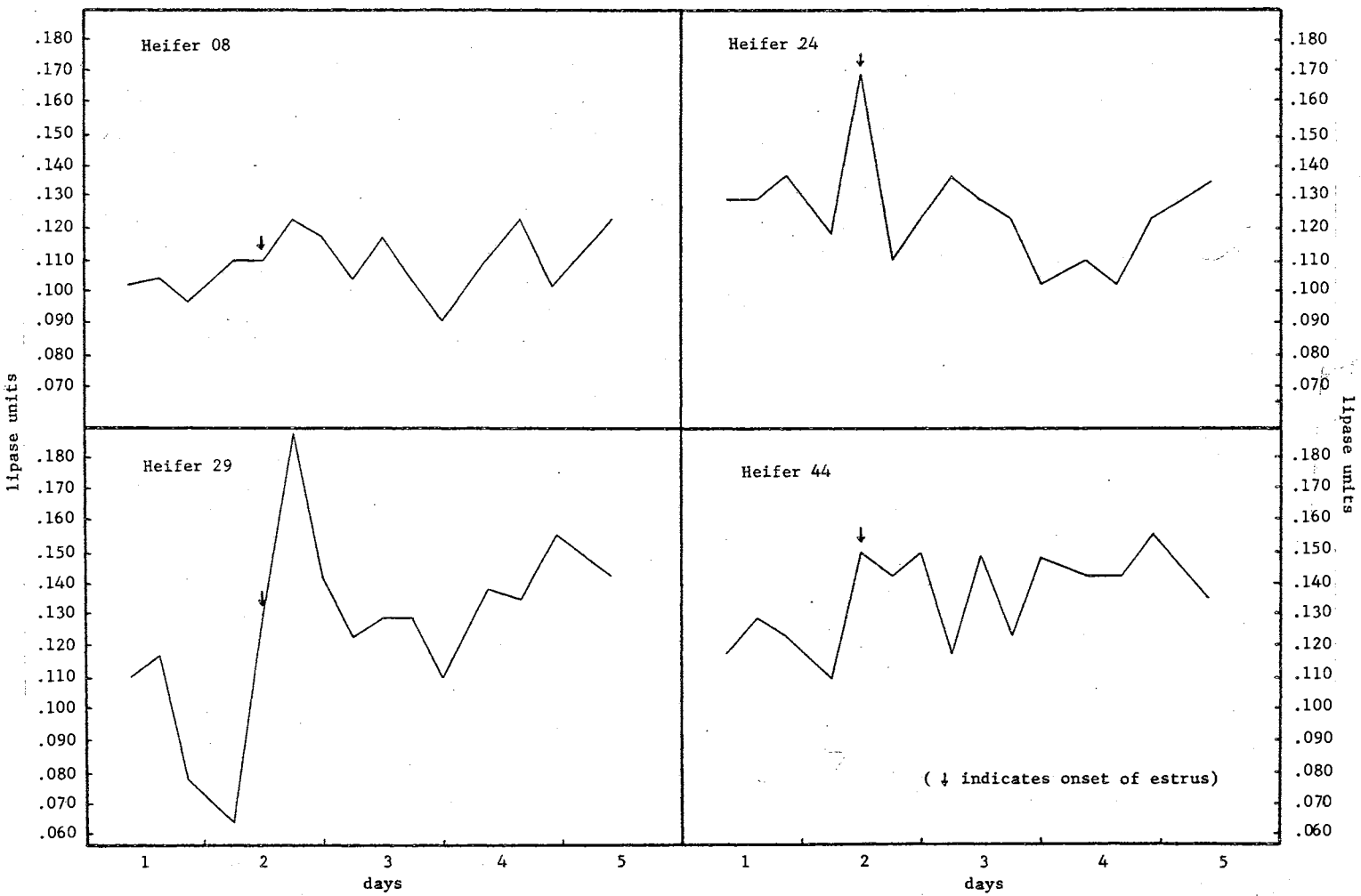

Figure 8. Lipase activity (lipase units per ml of plasma) of Heifers 08, 24, 29 and 44 during the second estrus following MGA treatment.

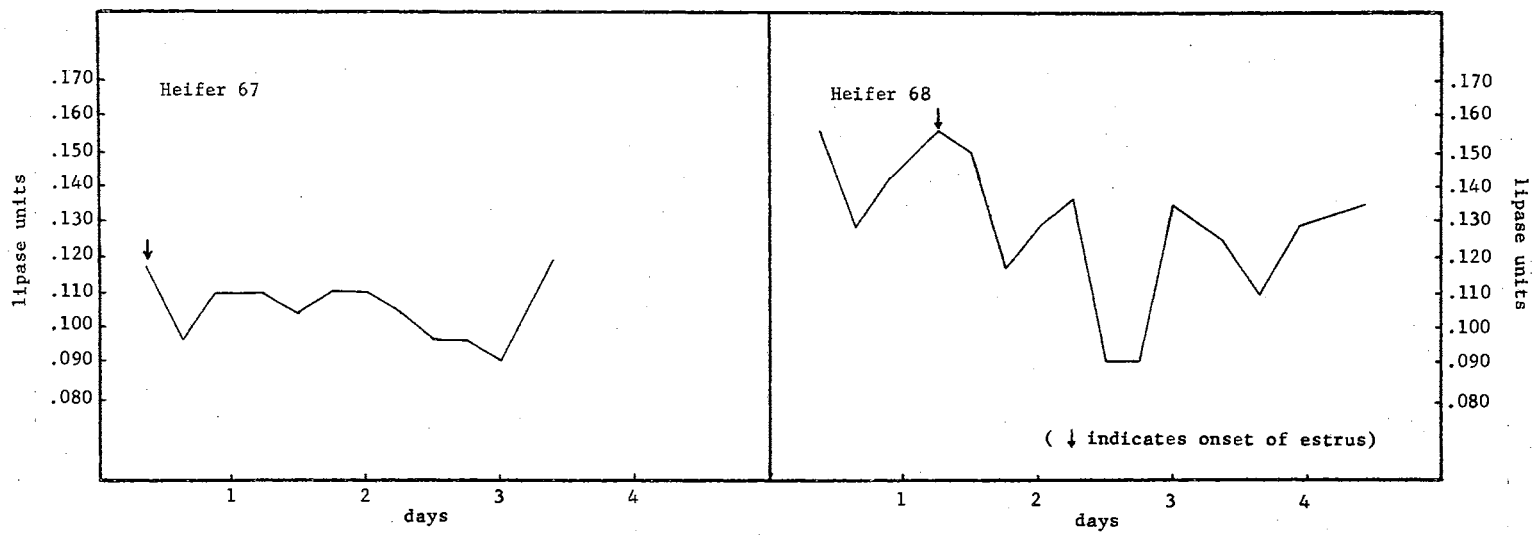

Figure 9. Lipase activity (lipase units per ml of plasma) of Heifers 67 and 68 during the second estrus following MGA treatment.

TABLE I

LIPASE UNITS AT ESTRUS AND SIX HOURS PRIOR TO ESTRUS FOLLOWING MGA REMOVAL

Animal Number	Pre-estrus	Estrus
24[a]	.104	.127
28[a]	.059	.098
29[a]	.087	.098
44[a]	.070	.104
67[a]	.093	.104
68[a]	.104	.110
08[b]	.110	.110
24[b]	.117	.168
29[b]	.064	.129
44[b]	.110	.149
68[b]	.142	.155

[a] first estrus following MGA removal
[b] second estrus following MGA removal

$t = 4.301$ ($P < .01$)

period following MGA treatment, the lipase activity of heifer 28 appeared to fluctuate over rather extreme ranges. It is interesting to note that within seven days after the onset of estrus, this animal again exhibited signs of heat. In all, three heat periods were detected over a 24 day period. Visual inspection of the ovaries after ovariectomy confirmed the presence of many large cystic follicles. No corpus luteum was visible indicating that ovulation had not occurred. In addition, the ovaries from heifer 41 contained several follicular cysts, probably accounting for her failure to exhibit heat during this period. The ovaries from the other six animals appeared normal with at least one corpus luteum visible for each pair.

The mean lipase activity values for the ovariectomized-controls and the ovariectomized-estradiol benzoate injected animals used in Phase 2 of Trial II are presented graphically in Figure 10. The relationship between the two groups is shown in a slightly different manner in Figure 11, where the mean lipase activity values are expressed as a percentage of the mean control values. This removes the variation in the control group by adjusting it to a constant value, so that the differences in the treated group may be seen more clearly. At the first sampling period on day 1, before the estrogen injections, the means of the two groups were approximately the same. Also, little difference was noted between the means of the two groups four hours after the initial injections. However, by the third sampling period, eight hours after the hormone injection, the lipase activity in the treated group had begun to increase. The mean values for the estrogen treated group were consistently higher than those of the control group for the rest of the first three days of treatment. There was little difference in

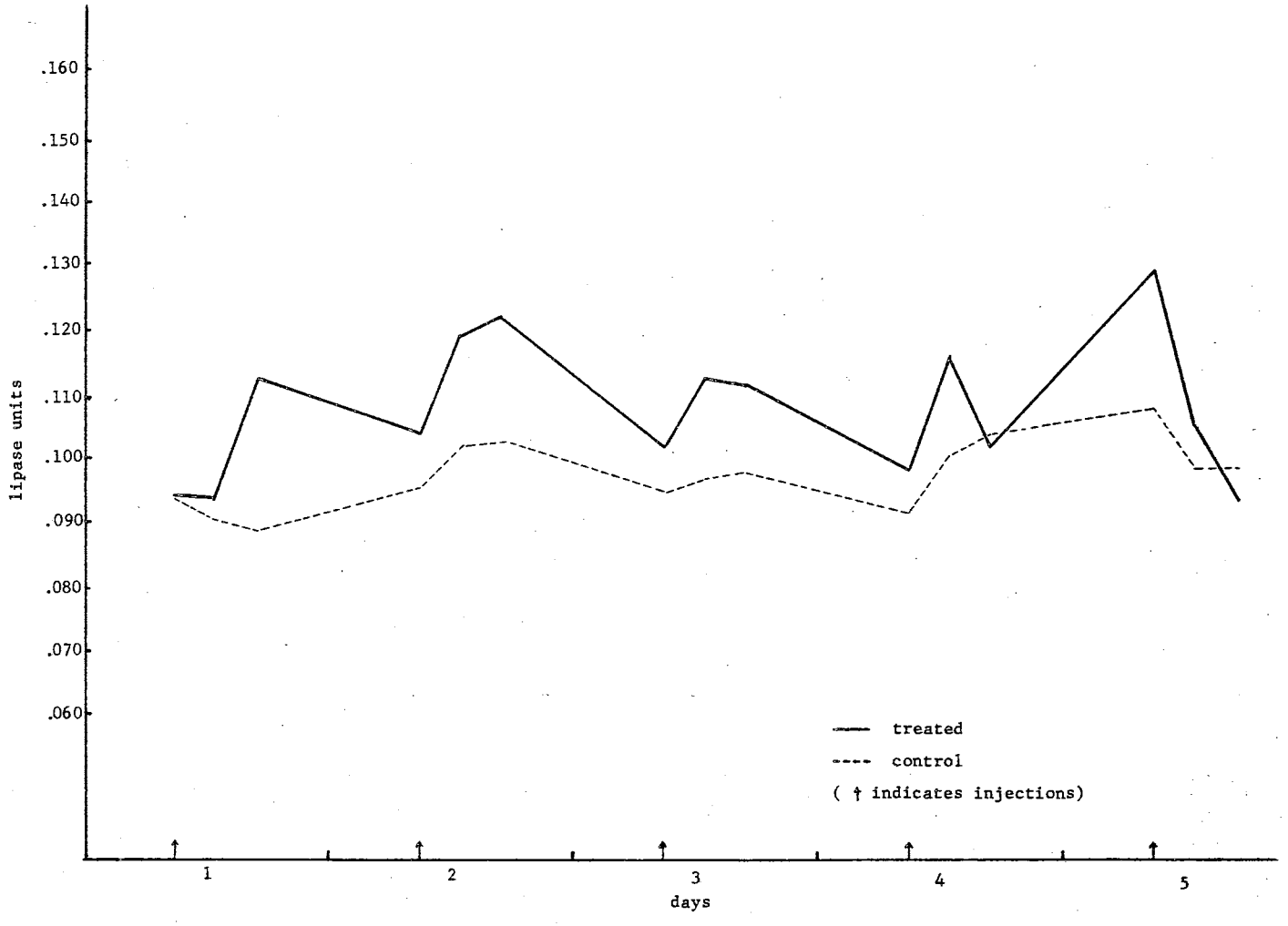

Figure 10. Mean lipase activity (lipase units per ml of plasma) for estrogen-treated and control animals measured three times daily during the five-day treatment period.

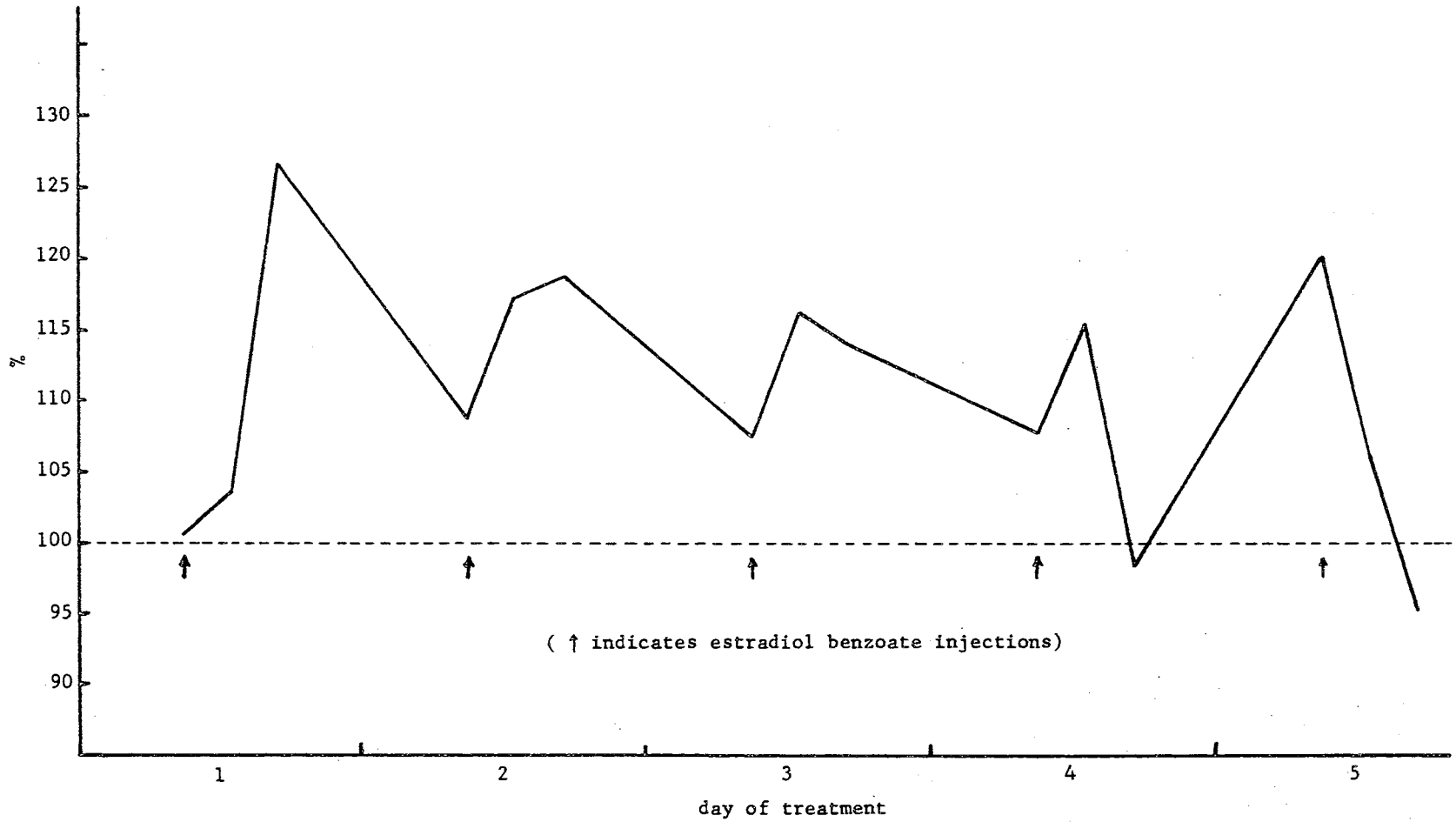

Figure 11. Mean lipase activity of estrogen-treated group during the five-day treatment period expressed as percentage of mean control values.

the means of the two groups of samples collected on the fourth and fifth day of treatment.

The daily changes in lipase activity for the treated and control groups are presented in Figure 12. This graph clearly expresses the daily effect of estrogen injections on the mean lipase activity values of the treated group. It appears that the initial effect of the hormone occurred between four and eight hours after the first injection. Following the injections on the second day, there was an immediate increase in mean lipase activity of this group. However, there was little change between four and eight hours post-injection. The pattern for the third day of injection was similar. On the fourth day there was an increase during the first four hours after the hormone injection and a decrease in activity during the second four hours. The fifth day of treatment showed a decline in activity following the injection, probably because of the high initial values of that day prior to the injections. The mean control values were also high at that time. The values for the control group remained fairly constant throughout the five-day period.

Statistical comparison of the two groups by analysis of variance for the five-day injection period is presented in Table II. From the pooled AOV, a direct comparison of the treated group and control group yielded an F value of only 1.62 which indicated that there was no significant effect of treatment. However, a significant effect due to time was noted. The individual analysis of variance for each group showed significant ($P < .005$) within group variation in each group. It should be noted that the time effect was significant in the treated group while it did not appear to be a factor in the control group. This indicates that the hormone injections caused a significant variation or

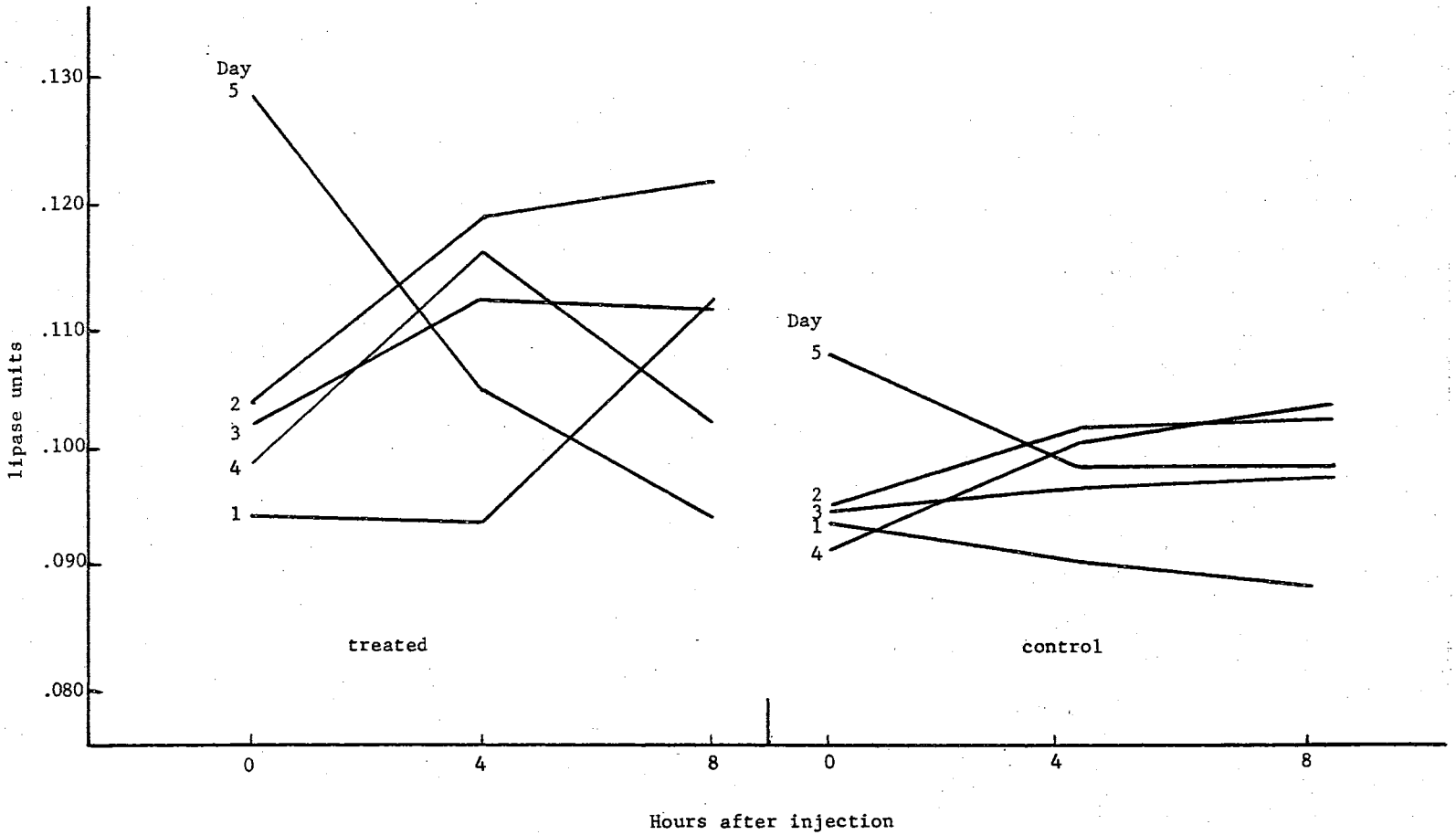

Figure 12. Daily variations in mean lipase activity (lipase units per ml of plasma) of estrogen-treated and control animals during the treatment period.

TABLE II

ANALYSIS OF VARIANCE OF LIPASE UNITS PER MILLILITER OF PLASMA COLLECTED FROM ESTROGEN-TREATED AND CONTROL ANIMALS DURING A FIVE-DAY TREATMENT PERIOD

Source	Pooled AOV d.f.	MS	F
Total	119		
Treatment	1	880.21	1.62
Animals in Treatments (Error a)	6	543.07	
Time in Treatments	28	80.68	
Hours in Days	14	119.11	2.88*
Hours	2	18.43	
Days	4	133.58	
Hours x Days	8	137.04	
Treatment x Hours in Days	14	42.26	1.02
Treatments x Hours	2	4.93	
Treatments x Days	4	18.96	
Treatments x Hours x Days	8	63.25	
Animals x Time x Treatments (Error b)	84	41.38	

AOV for Treated Group				AOV for Control Group			
Source	d.f.	M.S.	F	Source	d.f.	M.S.	F
Total	59			Total	59		
Animals	3	562.89	11.91**	Animals	3	523.26	14.74**
Time	14	131.02	2.77*	Time	14	30.35	.86
Animals x Time	42	47.26		Animals x Time	42	35.50	

* $P < .01$
** $P < .005$

fluctuation in lipase activity over a greater range, from one sampling period to the next, over the period of time in which the samples were collected. The variation between animals in each group, as well as the fact that there were only four animals in each treatment group, probably accounts for the failure to measure a significant difference between groups by this method. However, when the lipase activity units for the two groups were compared by analysis of variance for each individual sampling period, the estrogen-injected group were significantly ($P < .005$) higher eight hours after the first injection on day 1 than the control group.

Since the first sample was collected on day 1 before the hormone was injected and the data indicated that there was little obvious effect of the hormone during the last day of injection, the treated and control groups were statistically compared using the samples taken in the last two collections of day 1 and all the collections in days 2, 3 and 4. In order to remove the within-group variation of each group, only the mean lipase activity values for each group at each collection period were considered. The results shown on Table III indicated a highly significant ($P < .001$) difference between groups during this period.

The short duration of the estrogenic response was not surprising. The hormone-injected heifers began to show signs of heat during the second day of injections. This was indicated by swelling and vascularization or redness of the vulva, secretion of vaginal mucus, and response to genital manipulation. By late in the third day of the injection period these signs had subsided and on the fourth day they were no longer visible. Van Tienhoven (1968) states that continued estrogen ad-

TABLE III

MEAN LIPASE UNITS PER MILLILITER PLASMA COLLECTED FROM ESTROGEN-TREATED AND CONTROL ANIMALS FOR THE FIRST FOUR DAYS OF TREATMENT

Collection Period		Estrogen Treated	Control
Day	Hr	Mean ± S.D.	Mean ± S.D.
1	1	.094 ± .031	.091 ± .012
1	5	.113 ± .008	.089 ± .008
2	9	.104 ± .010	.095 ± .021
2	1	.119 ± .015	.102 ± .014
2	5	.122 ± .020	.103 ± .013
3	9	.102 ± .006	.095 ± .014
3	1	.112 ± .008	.097 ± .024
3	5	.112 ± .006	.098 ± .015
4	9	.099 ± .014	.092 ± .027
4	1	.117 ± .020	.104 ± .011
4	5	.102 ± .007	.104 ± .012

$t = 5.140$ ($P < .001$)

ministration causes the nervous system to become refractory to estrogen, and therefore will not produce continuous heat in the ovariectomized cow. Carrick and Shelton (1969) found that physiological levels of estradiol benzoate, from 121 to 132 ug, readily induced estrus in ovariectomized heifers and did not induce a state of refractoriness. However, a refractory state was produced by large doses of 10 mg estradiol benzoate. It is possible, therefore, that the mechanism(s) by which response was induced had lost its sensitivity to the estrogen by the time of the fifth injection.

The mean total extractable lipid values for each group at each collection period during the five-day hormone injection period are shown graphically in Figure 13. These values are means of duplicate samples from two animals from each group. The lipid values of the treated group are expressed as a percentage of control values in Figure 14. A large increase in total lipid was noted in the treated group within the first four hours after the first injection, and another increase of a smaller magnitude was found in the second four-hour period. The values for the control group also increased during this time and were in the same general range as the treated group. However, after the first estrogen injection, the mean total lipid values for the treated group were consistently higher than the mean values for the controls for the five-day treatment period. Statistical analysis of the data by comparing group means, paired for each sampling period, is presented in Table IV. A highly significant ($P < .001$) difference was found between the treated and control groups.

The levels of cholesterol esters, triglycerides, free cholesterol, diglycerides, monoglycerides and free fatty acids for the four animals

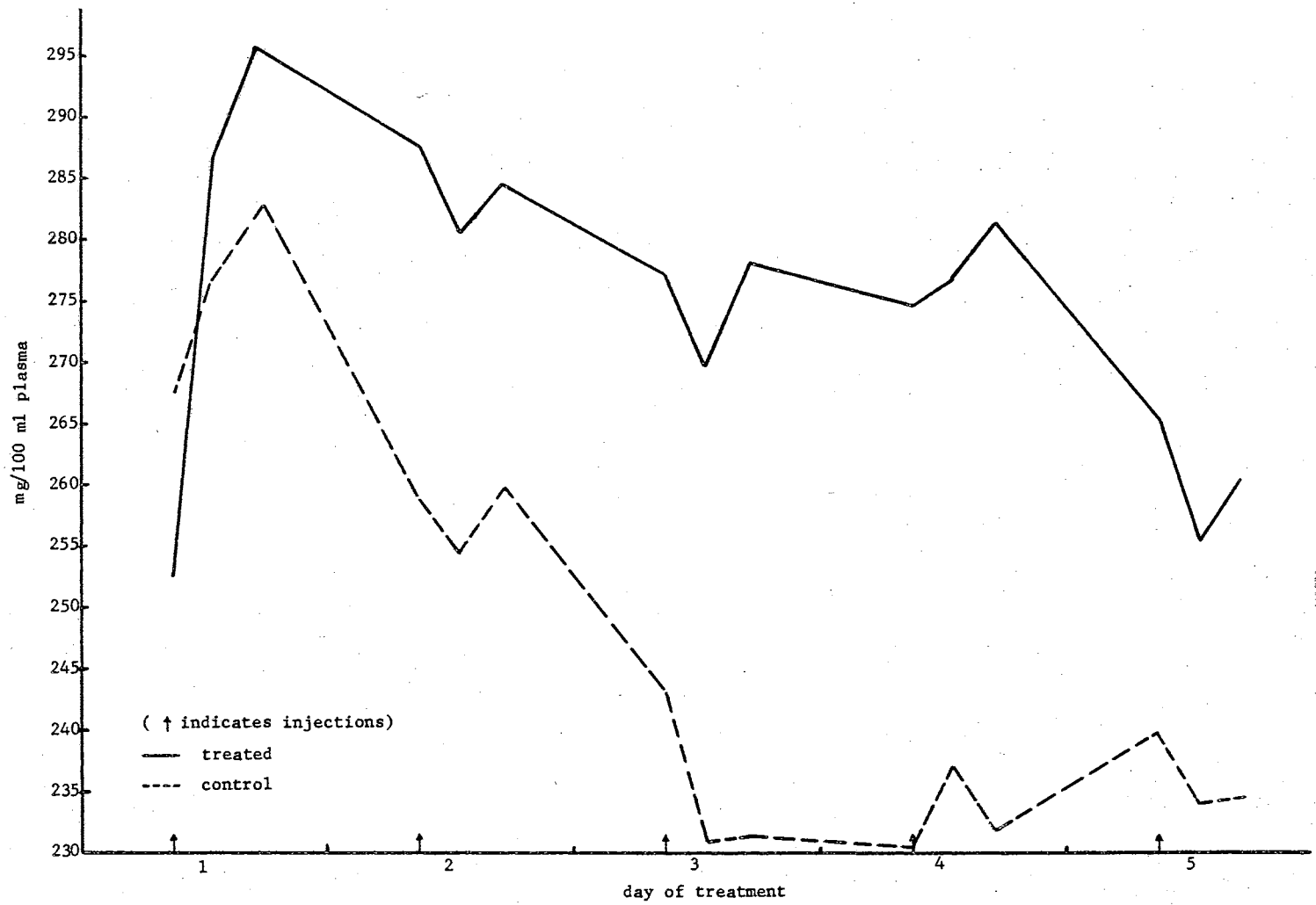

Figure 13. Mean total lipid levels of estrogen-treated and control animals measured three times daily during the five day treatment period.

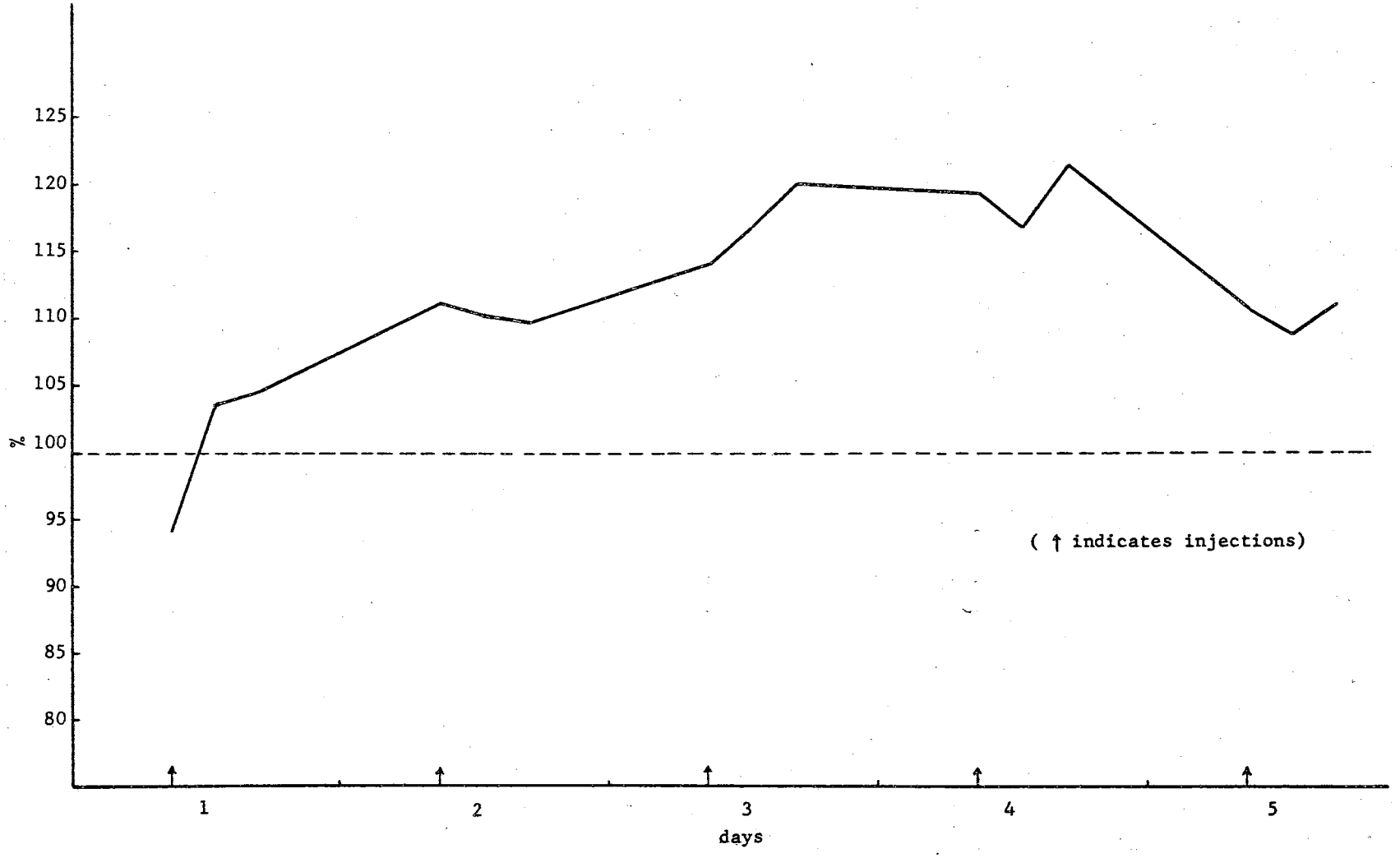

Figure 14. Mean total lipid levels of estrogen-treated group during the five day treatment period expressed as percentage of mean levels of the control group.

TABLE IV

MEAN TOTAL LIPID VALUES FOR ESTROGEN-TREATED AND CONTROL ANIMALS

Collection Period		Estrogen Treated	Control
Day	Hr	Mean* ± S.D.	Mean* ± S.D.
1	1	286.575 ± 36.8825	276.825 ± 21.3974
1	5	295.500 ± 9.8488	283.000 ± 5.0523
2	9	287.475 ± 19.8592	258.825 ± 5.6788
2	1	280.500 ± 28.6879	254.500 ± 11.0087
2	5	284.675 ± 41.3475	259.675 ± 14.9896
3	9	277.175 ± 31.3802	243.150 ± 10.0613
3	1	269.675 ± 38.5289	231.000 ± 15.5925
3	5	278.000 ± 41.0551	231.675 ± 11.3926
4	9	274.825 ± 38.7227	230.500 ± 6.4436
4	1	276.825 ± 42.9579	237.325 ± 7.8069
4	5	281.500 ± 46.4053	231.825 ± 4.6464
5	9	265.825 ± 37.5239	239.825 ± 9.9087
5	1	255.175 ± 42.2608	234.175 ± 7.2145
5	5	260.650 ± 37.7053	234.675 ± 14.6838

*mg lipid per 100 ml plasma
$t = 9.426$ ($P < .001$)

under study are presented in Figures 15, 16, 17, 18, 19 and 20, respectively. These figures show the levels of the individual fractions separated by the Florisil columns. Only the values for the first three days of treatment are presented because impurities in the ethyl ether which was used on the samples from day 4 and day 5 prevented accurate quantitation of the levels of the individual fractions. The graphs indicate that the levels of most of the fractions were quite variable from the day prior to the hormone treatment through the first three days of the treatment period. However, a definite change in the levels of the lipid fractions could not be ascribed to the estrogen treatment, since similar variations were noted in the levels of the fractions from the control animals. It appeared that the increase in mean total lipid values for the treated animals was largely accounted for by increased levels of cholesterol esters, primarily in heifer 67. A slight increase in triglyceride levels and a decrease in diglyceride levels were also noted. However, similar changes of a smaller magnitude were noted in the levels of these fractions in the control animals.

It was expected that changes in the levels of these lipid fractions would provide an indication of the function of possible simultaneous changes in plasma lipase activity. However, due to the small numbers of experimental animals used in this phase of the study, definite conclusions concerning changes in levels of lipid fractions and their relationship with lipase activity are impossible when natural animal variation exists. Similar work reported by Fewster et al. (1967) involved separation of plasma lipid fractions of the rat by silicic acid chromatography. They reported a significant reduction of total lipid, total cholesterol and monoglycerides following injection of es-

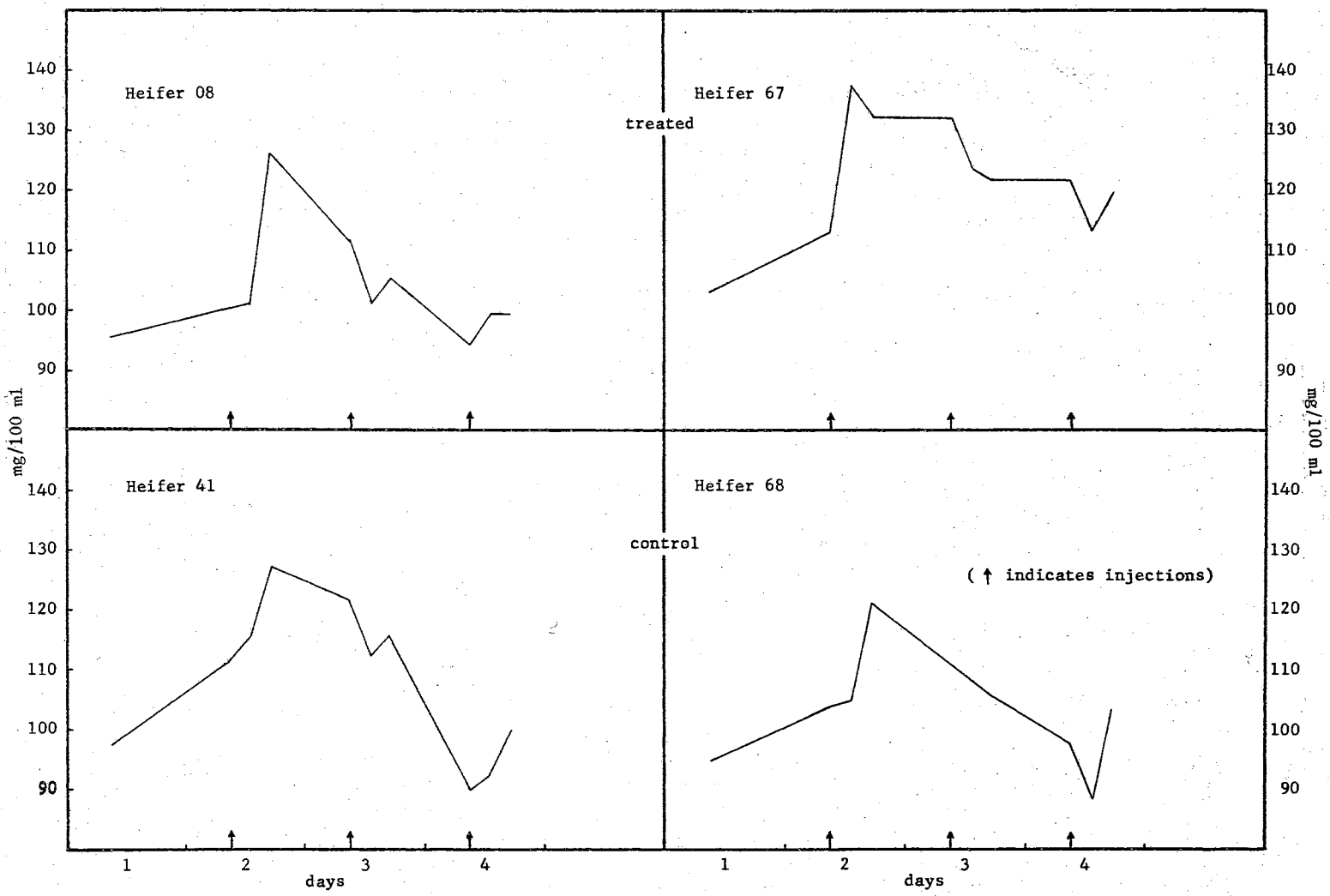

Figure 15. Levels of cholesterol esters (mg/100 ml plasma) for two estrogen-treated and two control animals during the first three days of treatment.

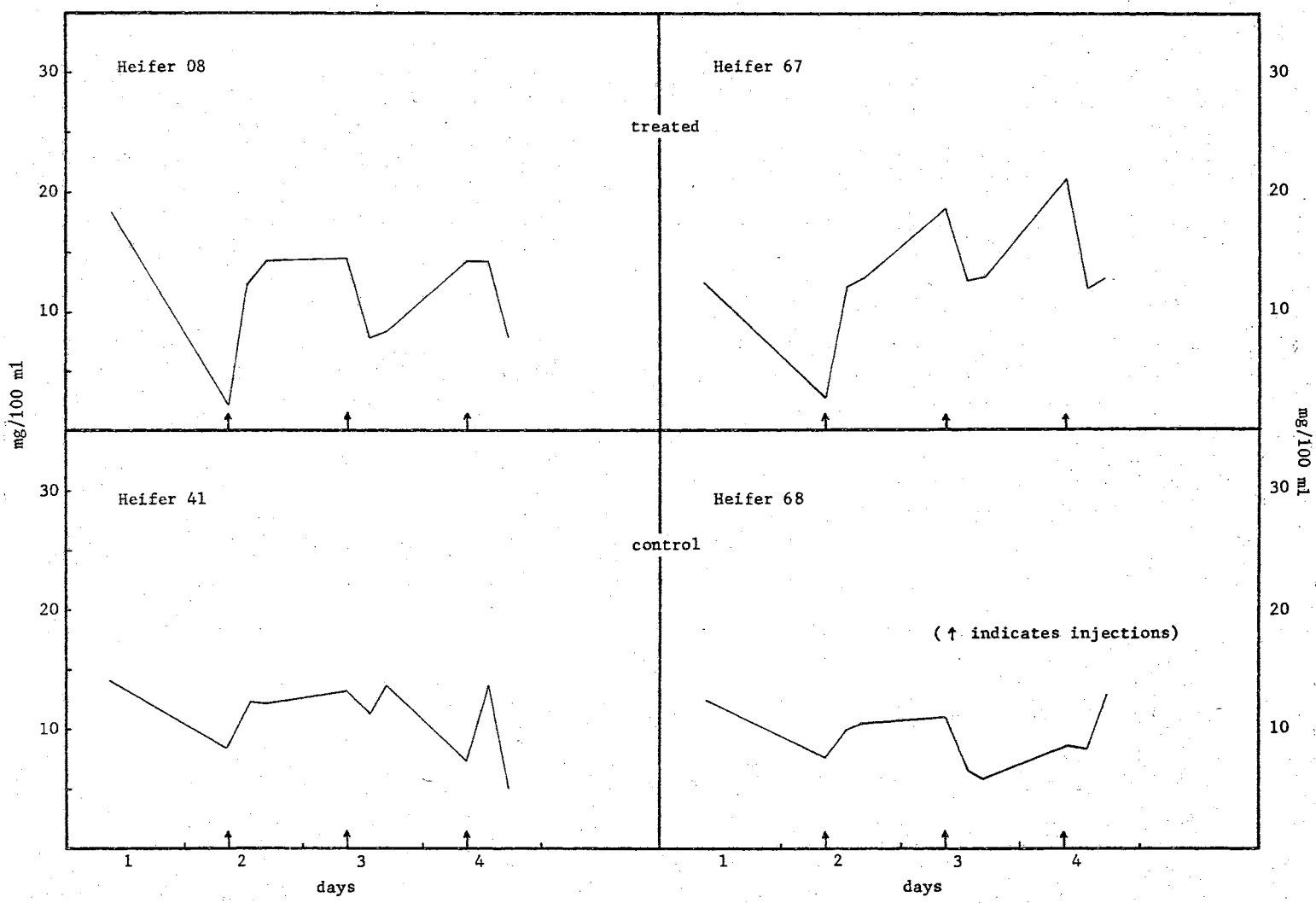

Figure 16. Levels of triglycerides (mg/100 ml plasma) for two estrogen-treated and two control animals during the first three days of treatment.

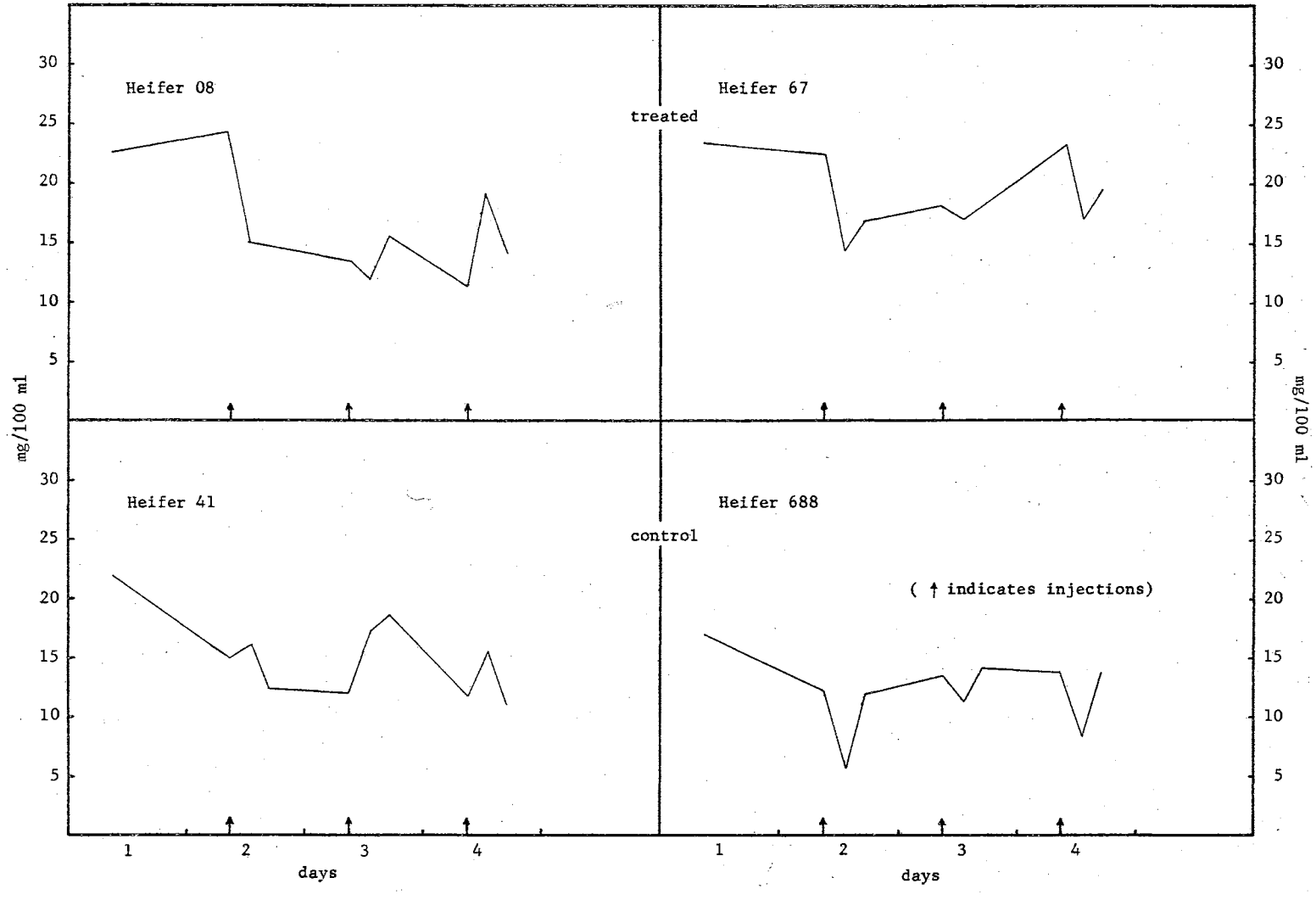

Figure 17. Levels of free cholesterol (mg/100 ml plasma) for two estrogen-treated and two control animals during the first three days of treatment.

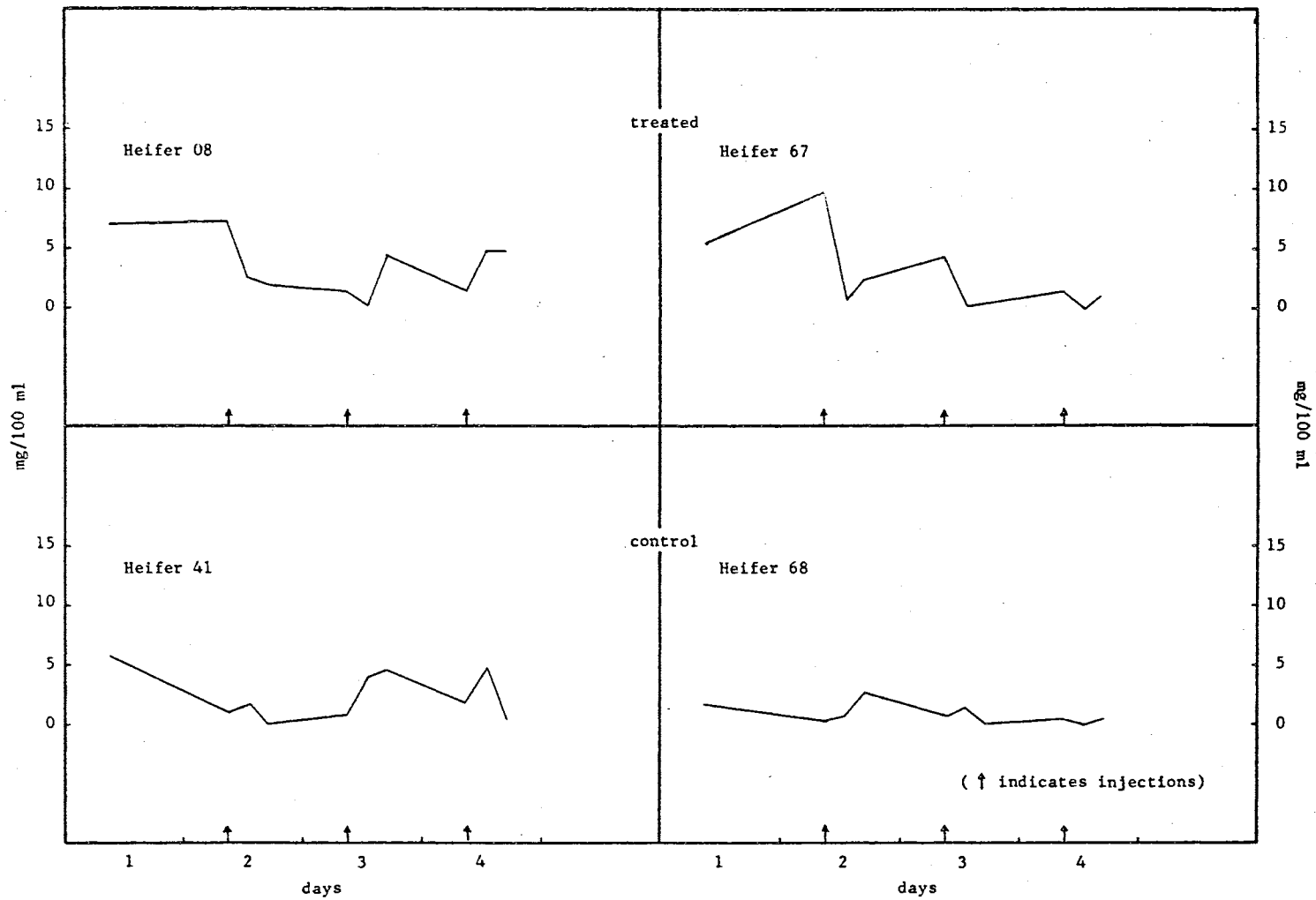

Figure 18. Level of diglycerides (mg/100 ml plasma) for two estrogen-treated and two control animals during the first three days of treatment.

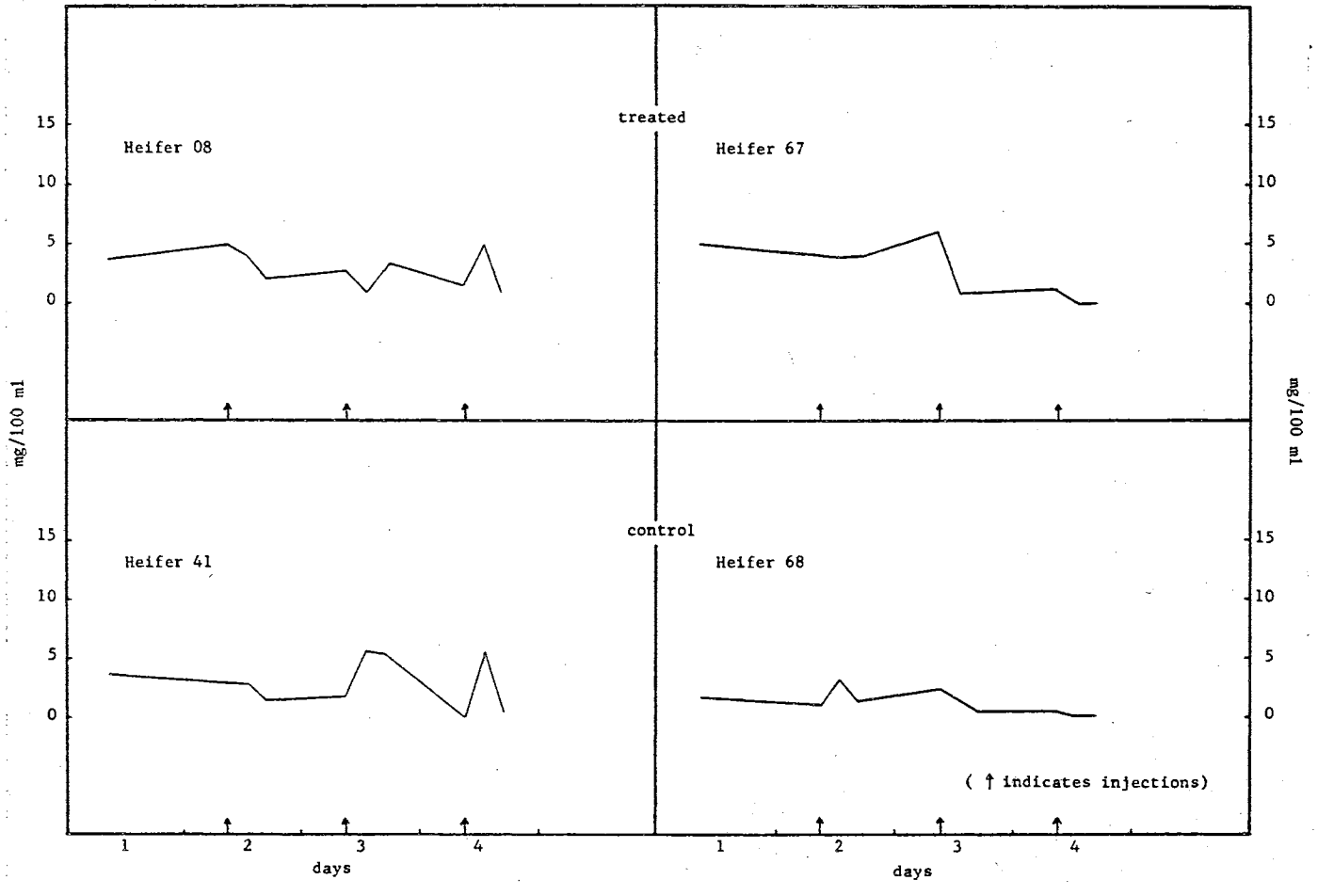

Figure 19. Levels of monoglycerides (mg/100 ml plasma) for two estrogen-treated and two control animals during the first three days of treatment.

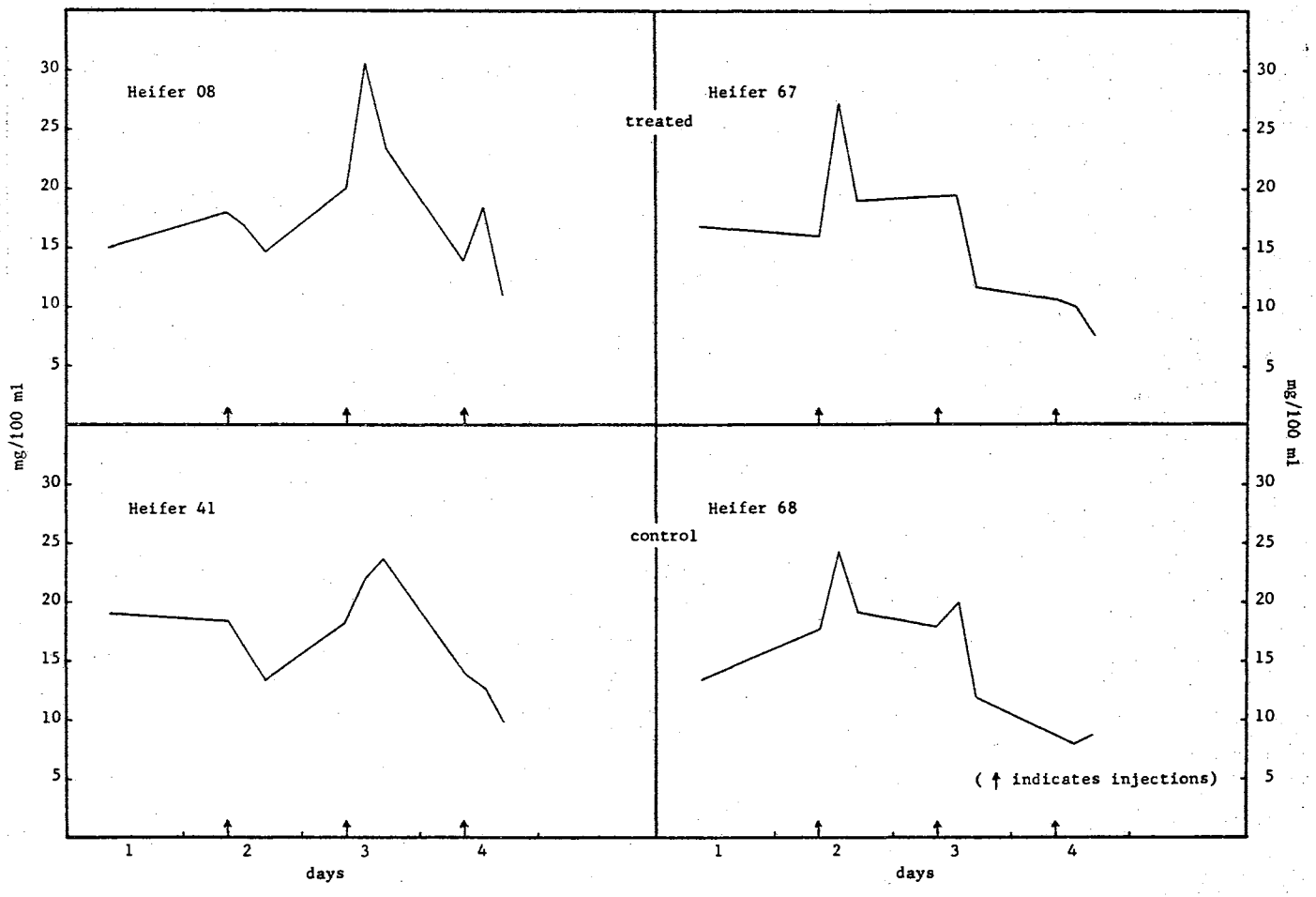

Figure 20. Levels of free fatty acids (mf/loo ml plasma) for two estrogen-treated and two control animals during the first three days of treatment.

tradiol benzoate. However, the values were obtained by pooling the plasma extracted from each group of five rats. Such a method did not account for the within-group variation between animals. The results of this study are not consistent with those reported above, indicating the possibility of a species difference in the effect of estrogen on lipid levels. However, a specific comparison between the studies is difficult because of possible variations in experimental conditions and the small number of experimental animals used in this study.

The results of this investigation support the previous reports of Haggerty (1966) and Wells et al. (1969) which indicate that the blood plasma lipase activity of the bovine can be expected to follow a consistent pattern during the estrogenic phase of the estrous cycle. Whether this estrogenic effect is a direct or indirect effect has not been determined. In addition, it is not known whether this lipase or lipase system functions in the mobilization or deposition of fatty acids in the tissue. The apparent increase in plasma total lipids would indicate a mobilization from the tissues or alteration in lipid uptake and metabolism by the tissues. Such an effect could be accomplished through stimulation of the hormone-sensitive lipase system. A direct effect of estrogen on this system has not been reported, however, some of the hormones which increase the activity of the enzyme can possibly be affected by estrogen levels. D'Angelo and Fisher (1969) have reported that physiological levels of estradiol promoted secretion of the thyroid-stimulating hormone in the rat. On the other hand, an estrogenic effect on the lipoprotein lipase system is also possible. Although a direct effect has not been reported, Dalderrup (1959) suggests that estrogens increase the plasma levels of heparin since an increase

of heparin occurs during the follicular phase of the human menstrual cycle. Heparin is the principle substance known to stimulate the release of lipoprotein lipase. Further research is necessary to determine the chemical nature of the lipase or lipase system measured in this study, the conditions under which it acts, and its function in the physiology of the mammalian system.

CHAPTER V

SUMMARY AND CONCLUSIONS

Six normal, cycling, non-lactating Holstein cows were used in Trial I to study the variations in blood plasma lipase activity during the estrous cycle. Blood samples were collected twice weekly for a period of five weeks, then daily for 15 days. At the first sign of estrus activity, each animal was bled at four-hour intervals for 24 hours. The resulting lipase activity patterns indicated a consistant peak of lipase activity within two days prior to the onset of estrus. This was followed by a sharp decrease at the beginning of estrus, then another peak of lesser magnitude during mid-estrus.

Eight normal, cycling Ayrshire heifers were used in Trial II to measure changes in lipase activity associated with estrus and following injections of estradiol benzoate. In order to measure changes in lipase activity prior to estrus, the heifers were fed the estrus synchronizing compound melengestrol acetate (MGA), at a rate of 1.0 mg per head per day for 15 days. Blood samples were collected three or four times daily for eight days following MGA removal. Lipase activity patterns during the first and second heat periods after MGA treatment in most cases were similar to those observed in Trial I. A significant ($P < .01$) increase in lipase activity was observed during the six hour period prior to the onset of estrus, from a mean of .096 to a mean of .123 lipase units per ml plasma.

Phase 2 of this trial was concerned with the investigation of the effects of estradiol benzoate on blood plasma lipase activity and levels of circulating lipids. The eight heifers were ovariectomized and randomly assigned to a treated or control group. Each of the four animals in the treated group received daily injections of 5.0 mg estradiol benzoate dissolved in 1.0 ml ethyl oleate for five days. The four animals in the control group received similar injections of the carrier substance without the hormone. Blood samples were collected each day before the injection and at four and eight hours post-injection. Analysis of variance indicated that the lipase activity values for the treated group were not significantly different from the control group for the five-day treatment period. However, when the variation between animals was removed by comparing group means for each sampling period, a highly significant ($P < .001$) difference was found between the means of the treated and control groups during the first four days of treatment. In addition, comparison of means of total plasma lipid from two animals from each group indicated that the plasma lipid means of the treated group were significantly ($P < .001$) higher than those of the controls. Comparisons of plasma lipid classes separated by column chromatography on Florisil columns were inconclusive, due to the small numbers of animals used and the variation between animals in each group.

This study indicated that blood plasma lipase activity is affected by the estrogenic phase of the bovine estrous cycle. Although other factors influence the activity of the enzyme during the estrous cycle, the relatively high levels of estrogens during late proestrus and estrus appear to cause a consistent pattern of lipase activity. Since estrogen injections in ovariectomized animals resulted in a significant

increase in lipase activity, this hormone apparently is at least one of the factors affecting the pattern of lipase activity during this phase of the estrous cycle.

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VITA

2

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